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Biotechnology applied to aromatic plants for the controlled production of bioactive compounds

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DECLARATION

I, Roméo Arago Dougué Kentsop, declare that this thesis is my own work except where acknowledged. It is being submitted for the Degree of Doctor of Philosophy at the University of Genoa (Italy). It has not been submitted before for any degree or examination at any other University.

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(Signature of candidate)

……..day of ……..2020

This thesis is dedicated to my family Dougué for their love and support.

ABSTRACT

Salvia sensu lato includes more than about 9000 species and is considered one of the largest taxa of the Lamiaceae (Will et al., 2015). For a long time, *Salvia* species were known for a wide variety of medicinal used in folk medicine for the relief of pain, protecting the body against oxidative stress, free radical damages, angiogenesis, inflammation, bacterial and virus infection, etc… (Hamidpour et al., 2014). *In vitro* cultures have been considered as an alternative agricultural processes for producing secondary metabolites (Y. Kim et al., 2002). The research activity of this PhD project was aimed to the application of *in vitro* biotechnology techniques applied to aromatic plants for the production of bioactive compounds. The selected plants are species of *Salvia* showing an important antibacterial activity. The investigation study was carried out on *S. corrugata* and *S. tingitana*.

Salvia corrugata Vahl. is an ornamental plant that grown easily along the Mediterranean coast. The aerial part is rich in terpenoid compounds like the diterpene quinones fruticuline A and demethylfruticuline A that present high antibacterial activity. Protocols of tissue culture and genetic transformation were set up with the aim to obtain *in vitro* shoot and hairy root biomass to grown in controlled condition for metabolite extraction. Two strains of *Agrobacterium rhizogenes* (wild type ATCC 15834 and hypervirulent LBA9402) were tested for their ability to induce hairy root on wounded leaves. The best response (75 %) was achieved by infection with ATCC 15834 about thirty days after the infection onto the hormone-free MS basal solid medium. Two hairy-root lines from ATCC 15834 treatment and one from LBA 9402 treatment were established. Transformation of selected clones was confirmed by polymerase chain reaction analysis of bacterial *rolC* and *virC1* genes. The growth evaluation in TIS RITA® bioreactor put in evidence the best cultural conditions for the biomass production; the clone SCO-HR-FA8 had the best increase on Murashige and Skoog (1962) and ½ Woody plant medium (Lloyd et al., 1980) salt compositions in comparison to other tested media. 30 mg/L was the best sucrose concentration that guaranteed the highest biomass production. The growth curve of HR lie SCO-HR-FA8 was elaborated and then, three classes of elicitors and their combination were tested: a heavy metal ions (Ag^{+}), the yeast extract and plant response-signaling compound (methyl jasmonate MJ). Among them, Ag+ and yeast extract (YE) at high concentration were

most effective to stimulate the biomass production. The scale up of the biomass was performed using the bioreactor RITA®. The methanolic extracts of the biomass (16.8 g) was fractionated by Si gel MPLC to obtain 16 fractions. The methanolic extract and the semi-purified fractions were tested against several multidrug resistant clinical strains of various bacterial species: *Staphylococci* and *Enterococci* and *E. coli*. The total extract was poorly effective while the semi purified fractions displayed variable potency with MIC values ranging from 8 to >128 µg/mL and from 4 to >128 µg/mL respectively against the *Staphylococci* and *Enterococci* strains considered. Our results suggest that the application of biotechnology approach on *S. corrugata*, it is possible to induce hairy roots from leaf, optimize the condition to increase the production of biomass, scale-up using TIS bioreactor and produce secondary metabolites with antibacterial activity.

The study carried out on *S. tingitana* aimed to develop an *in vitro* culture protocol to produce different plant tissues and investigate the antibacterial activity of the extract. The callus formation from sterilized leaves was evaluated on MS medium added with different PGRs in presence of ascorbic acid in light or in dark conditions. The result showed the importance of the presence 2,4-D and darkness for callus development. The high development percentage (94.4 %) was achieved by combining KIN and 2,4-D at the concentration of 0.5:0.5 or 1:1 mg/L respectively. The optimization of the medium for the callus growth was determined evaluating the type and concentration of cytokinin and auxin in dark. The suitable condition for callus proliferation was MS medium supplemented with 2,4-D 4.52 µM, KIN 2,32 µM and 10 mg /L of ascorbic acid. The elicitation with MJ or light intensity was investigated. The methanolic extract and fractions obtained by MPLC were inactive against *Staphylococci* species and *E. coli*. The fractions 11 and 12 displayed an antibacterial activity only against *E. faecalis* and *E. faecium* with MIC values ranging from 32 to 64µg/mL.

In *S. tingitana,* a successful protocol for callus production was established; this *in vitro* culture system can be used as good method to obtain medicinally-useful secondary compounds from *S. tingitana.*

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LIST OF ACRONYMS AND SYMBOLS

%: Percent

° C: Degree Celsius

µE m^{-2} s^{-1}: MicroEinsteins per m^2 per second

2,4-D: 2,4-Dichlorophenoxyacetic acid

Ag: Silver

ANOVA: One-way analysis of variance

ATCC: American Type Cell Culture

BA: Benzyl adenine

Cor: Coronatine

CT: cryptotanshinone

DT-I: dihydrotanshinone I

DNA: Deoxyribonucleic acid

EM: Extract of mycelium

EtOH: Ethanol

eV: Electron volt

g/L: gram per liter

g: gram

HPLC: High performance liquid chromatography

IBA: Indole-3-butyric acid

KIN: Kinetin

LC-MS: Liquid chromatography coupled with mass

m/z: mass to charge ratio

mg/L: Milligram per litre

mg: Milligram

MIC: Minimum inhibitory concentration

min: Minute

MJ: Methyl jasmonate

mS/cm: Millisiemens per centimeter

MS: Mass spectrometry

MS: Murashige & Skoog (1962)

MS0: Murashige & Skoog hormone-free

MTP: Meta-Topolin

NAA: 1-Naphthaleneacetic acid

NB: Nutrient broth

PCR Polymerase chain reaction

PGR: Plant Growth Regulator

pH: Potential hydrogen

PSF: Polysaccharide fraction

Q-TOF quadrupole- time of flight

Rf: Retention factor

Rpm: Rotations per minute

Rt: Retention time

S.D.: Standard deviation

S.E.: Standard error

SA: Salicylic acid

TAE: Tris-acetate-EDTA

T-I: Tanshinone I

T-IIA: Tanshinone IIA

TLC: Thin layer chromatography

TT: Total tanshinones

UV: Ultraviolet

V: Volt

WHO: World Health Organization

WPM: Woody Plant Medium

YE: Yeast extract

YMB: Yeast Mannitol Broth

µL: Microlitre

µM: Micromolar

CHAPTER 1 INTRODUCTION

Plants are a precious source of a huge variety of secondary metabolites such as steroids, alkaloids, flavonoids, terpenoids, anthocyanins, quinins, and lignans, which have a number of valuable utilizations in various industrial sectors as drugs, flavours, agrochemicals, dyes, fragrances, insecticides, biopesticides, as well as food additives (Khan et al., 2018).

According to the World Health Organization (WHO), 80% of people still relies on plant-based traditional medicines for primary health care and 80% of the 122 plant-derived drugs are related to their original ethno-pharmacological use (Dias et al., 2012). The current climatic and economic scenario pushes toward the use of sustainable resources to reduce our dependence on petrolchemicals and to minimize the impact on the environment. Plants are precious natural resources, because they can supply both phytochemicals and lignocellulosic biomass (Andre et al., 2016). Biotechnological approaches are increasing for medicinal plant biomass growth and secondary metabolite production. Current-day pharmaceuticals are typically based on plant-derived metabolites, with new products being discovered constantly. Nevertheless, the consistent and uniform supply of plant pharmaceuticals has often been compromised (Espinosa-Leal et al., 2018) by the modern city under construction, the increasing need for natural resources, the serious pollution, the environment has been never so tough for the growth of medicinal herbs. In another word, the collection in nature of the medicinal herbs can't fulfill the need of increasing market demand. The naturally grown medicinal herbs have now the risk to carry more and more herbicide, insecticide and heavy metals, which will cause contamination to the extract and finally cause side effects. Furthermore, due to the complex structures of secondary metabolites, the chemical synthesis is proved to be cost-inefficient in most cases. Thus, to find ways to produce enough medicinal herbal material in an appropriate manner becomes more and more urgent for the development of pharmaceutical industry all over the world (Gaosheng et al., 2012). In conclusion, in alternative to wild collection or plant culture in field, the production of useful and valuable secondary metabolite in large-scale from tissues grown in controlled conditions free from the climate and geographical constraints, is an attractive proposal; it should contribute significantly to future attempts to preserve global biodiversity and alleviate associated ecological (Espinosa-Leal et al., 2018; Ruffoni et al., 2010). Therefore, the answer for the production of important plant active compounds is to use *in vitro*

systems for shoot (micropropagation) cell and tissue culture. In particular, micropropagation allows the production of plant with uniform quality for agronomic culture and also for metabolite development under controlled condition; two types of *in vitro* cultures are currently used for secondary metabolite production: undifferentiated cultures (callus and cell suspension cultures) and differentiated organ cultures (hairy root and shoots) (Giulietti et al., 1997).

1.1. *In vitro* plant tissue culture

1.1.1. Historical background of plant tissue culture

The technique of *in vitro* cultivation of plant material began in the early 1900s by Haberlandt who, based on the concept of totipotency of plant cells (Haberlandt, 1902), resumed and expanded the cellular theory formulated by Schwann and Schleiden 60 years earlier which suggested that "every structural part of a plant was made up of cells or the result of cells". Since this moment up today, many other interesting discoveries were accomplished on the plant world: Went et al. (1937), discovered the first plant hormone, indole-3-acetic acid (IAA); Miller et al. (1955), isolated the first cytokinin (kinetin or 6-benzyl-aminopurine) from the degradation products of the autoclaved DNA and demonstrated its function on promoting cell division in cultures of tobacco calluses. In 1962 Murashige & Skoog, (1962), developed a particular balanced macroelements and microelements salt composition for the substrate preparation always referred as "Murashige and Skoog", which is still the most used medium in the field of *in vitro* plant cultures. In 1977, Chilton et al. (1977), successfully integrated a Ti plasmid DNA from *Agrobacterium tumefaciens* in plant cells and later, Horsch RB et al. (1985) regenerated transgenic tobacco plants. Nowadays, *in vitro* culture techniques are useful tool to improve production and marketing of plant species allowing to make a rapid clonal propagation of plant selected for their active principles (Lucchesini et al., 2010).

1.1.2. Base concept of plant cell and tissue culture

Plant tissue culture combines a series of techniques useful to set up a sterile culture in vitro, to maintain and proliferate the tissues in controlled conditions on a nutrient culture medium of known composition (Hussain et al., 2012).

Sterilization: The surface sterilization of explants in chemical solutions is an important step to remove contaminants with minimal damage to plant cells. The disinfectants most commonly used are sodium hypochlorite, calcium hypochlorite, ethanol, and mercuric chloride ($HgCl_2$). The instruments are most often sterilized by heating by flame or autoclave. The culture vessels containing the culture medium as for them are sterilized in autoclave. The aseptic manipulations are then carried out in a laminar flow hood, which was previously thoroughly wiped out with rectified spirit.

Culture medium or substrate: Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients (Mg, Ca, P, S, N and K), micronutrients (Fe, Cu, Mn, Co, Mo, B, I…), vitamins, other organic components, natural or synthetic plant growth regulators (auxin, cytokinin), a carbon source (sucrose is commonly used) and some gelling agents (agar) in case of "solid" medium. The composition of the most commonly used medium Murashige and Skoog, 1962 is reported in (**Annex 1**). However, based on the qualitative and quantitative composition of the main compounds, different other types of substrate have been set up for different species or tissues considering the objective of the culture. The pH of the media is also important, it is generally adjusted (with 1.0 or 0.1 *N* HCl or NaOH) to pH 5.5 to 6 (T Murashige, 1973).

Physic factors: the development of *in vitro* explants is also influenced by light quality, intensity, photoperiod, and temperature in the growth chamber. Standard conditions are 20-25°C with 16/8 (light/dark) photoperiod with light intensity of 3000-5000 lux supplied by white fluorescent lamp.

1.1.3. *In vitro* culture techniques

- **Micropropagation or *in vitro* shoot propagation**

Micropropagation is the practice of multiplying stock plant material to produce a large number of clonal plants in relatively short time. It starts with the selection of the explants, generally, this process can be divided in five basic stages (from 0 to 4) (T. Murashige, 1974).

Stage 0: the mother plant should be *ex vitro* cultivated under hygienic conditions principally in greenhouse to minimize further contamination.

Stage 1 - sterilization: this stage involves the selection of explants, surface disinfection and cultivation under aseptic conditions. The cultures are incubated in growth chamber either under light or dark conditions according to the specific method of propagation (Hussain et al., 2012).

Stage 2 - multiplication: the aim of this phase is to increase the number of propagules (shoots) through repeated subcultures under aseptic conditions and it is generally mediated by cytokinins.

Stage 3 - rooting: it is the last step *in vitro* before transferring the developed plantlets to *ex vitro* conditions. This stage may occur simultaneously in the same culture medium used for explant multiplication. However, in some cases it is necessary to change medium, including nutritional modification and growth regulator composition to induce rooting and the development of strong root growth; it is normally auxin-mediated.

Stage 4 - acclimatization: the plantlets are transferred to ex vitro condition in the greenhouse.

- **Somatic embryogenesis and organogenesis**

Somatic embryogenesis is the process of a single or a group of totipotent somatic cells that begin the developmental pathway that leads to neoorganogenesis of bipolar structures able of germinating to form complete plant. It can be initiated directly from the explants or indirectly by the establishment of mass of unorganized cells called callus (Hussain et al., 2012). Plant

growth regulators, as mainly, 2.4-D and abscisic acid, play an important role in the regeneration and proliferation of somatic embryos.

Organogenesis refers to the production of plant organs i.e. roots, shoots and leaves that may arise directly from the tissues or indirectly from the undifferentiated cell masses (callus). With high auxin/low cytokinin roots develop, with low auxin/high cytokinin shoot buds develop; at intermediate levels undifferentiated callus tissue develops (Skoog et al., 1957).

1.1.4. *In vitro* culture for secondary metabolites production

The yield of useful secondary metabolites is often less than 1% dry weight and depends on the physiological and phenological stage of the plant either in field culture or in wild collection. In addition, many plants containing high-value compounds are difficult to cultivate or are becoming endangered because of overharvesting (Namdeo, 2007). The alternative is the biotechnological approach referred to cell or tissue culture in controlled conditions; these systems have potential as a complement of the production of the raw material or in some cases they are the alternative to the traditional agriculture in the industrial production of bioactive plant metabolites (Y. Kim et al., 2002; S Ramachandra Rao et al., 2002). Plant cell and organ culture systems are feasible option for the production of secondary metabolites that are of commercial importance in pharmaceuticals, food additives, flavors, and other industrial materials (Poornananda M Naik et al., 2016). However, the production of some secondary metabolites is related to the type of differentiation obtained *in vitro*; It is reported that *in vitro* production of secondary metabolites is much higher from differentiated plant organs (hairy roots, shoots) when compared to non-differentiated or less differentiated tissues as callus or cell culture alone (Kolewe et al., 2008; Tiwari et al., 2015). In order to increase yield it is possible, *in vitro*, to apply strategies such as elicitation, precursor feeding and metabolic engineering.

- **Plant cell culture**

The plant cell cultures provide a platform for the production of high-value secondary metabolites and other substances of commercial interest. Cell culture begin by sterilization of different types of plant somatic tissues (leaf lamina, petioles, roots) and their transfer onto a

nutrient medium containing plant growth regulators (Hall, 2000). The first action is the regeneration, on the surface of the cultured tissues, of calli that are clusters of dedifferentiated cells. These calli could be hard or friable, the last are the best source of cells that can be disrupted in liquid medium under constant shaking; a graphic representation of the cell culture cycle for metabolite production is summarized in **Fig. 1.1.** The cells are maintained in this state thanks to the use of phytoregulators in the growth substrate and medium is renewed weekly. However, plant cell culture can also be obtained through transformation with *Agrobacterium tumefaciens* (H. Chen et al., 1997; Moldenhauer et al., 1990; Song et al., 2000), in this case, the presence of growth regulators is not needed.

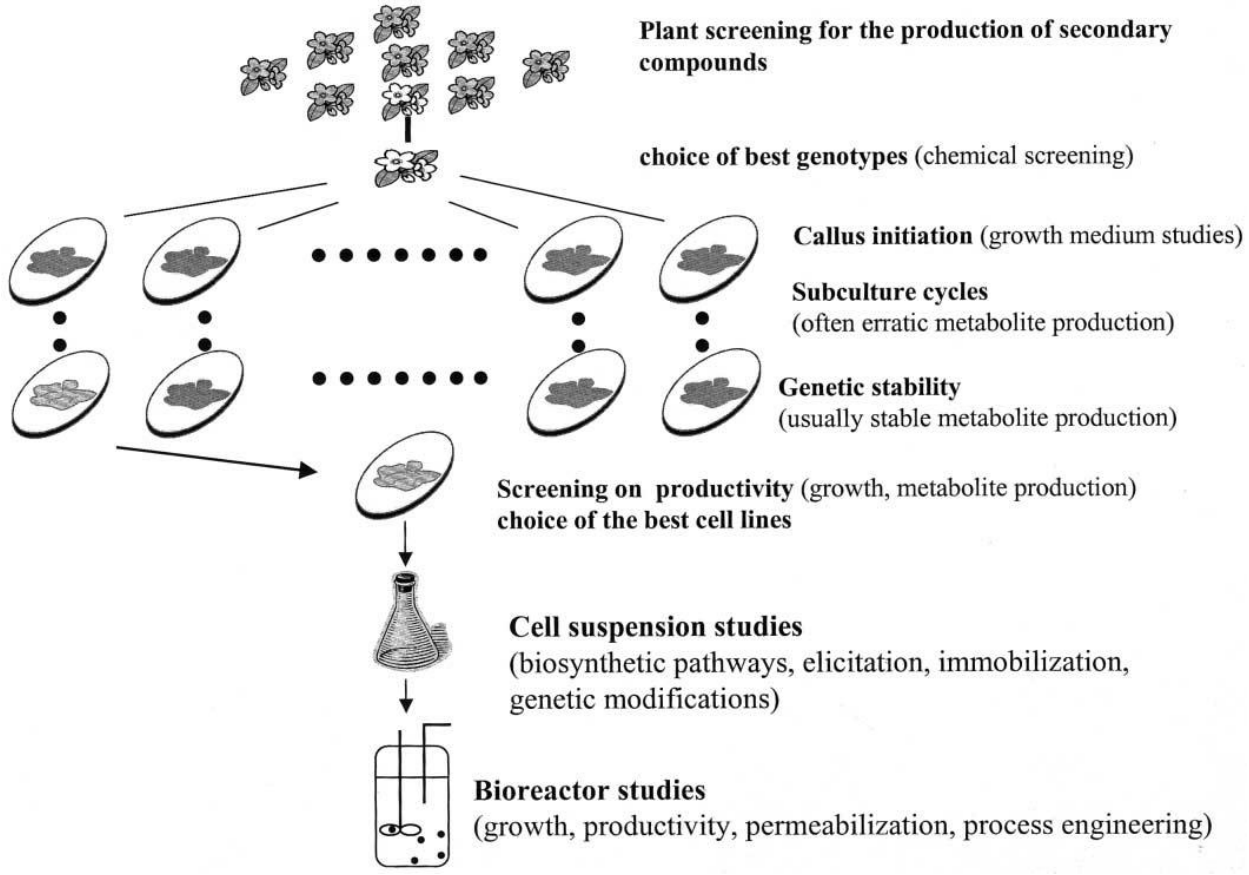


Figure 1. 1 Guidelines for the production of secondary metabolites from plant cell. (Bourgaud et al., 2001).

- **Plant organ culture**

Plant organs as shoots or hairy roots, represent an alternative to cell cultures for the production of plant secondary products; in **Fig. 1.2** there are summarized the principal steps for their direct or indirect (via callus) development.

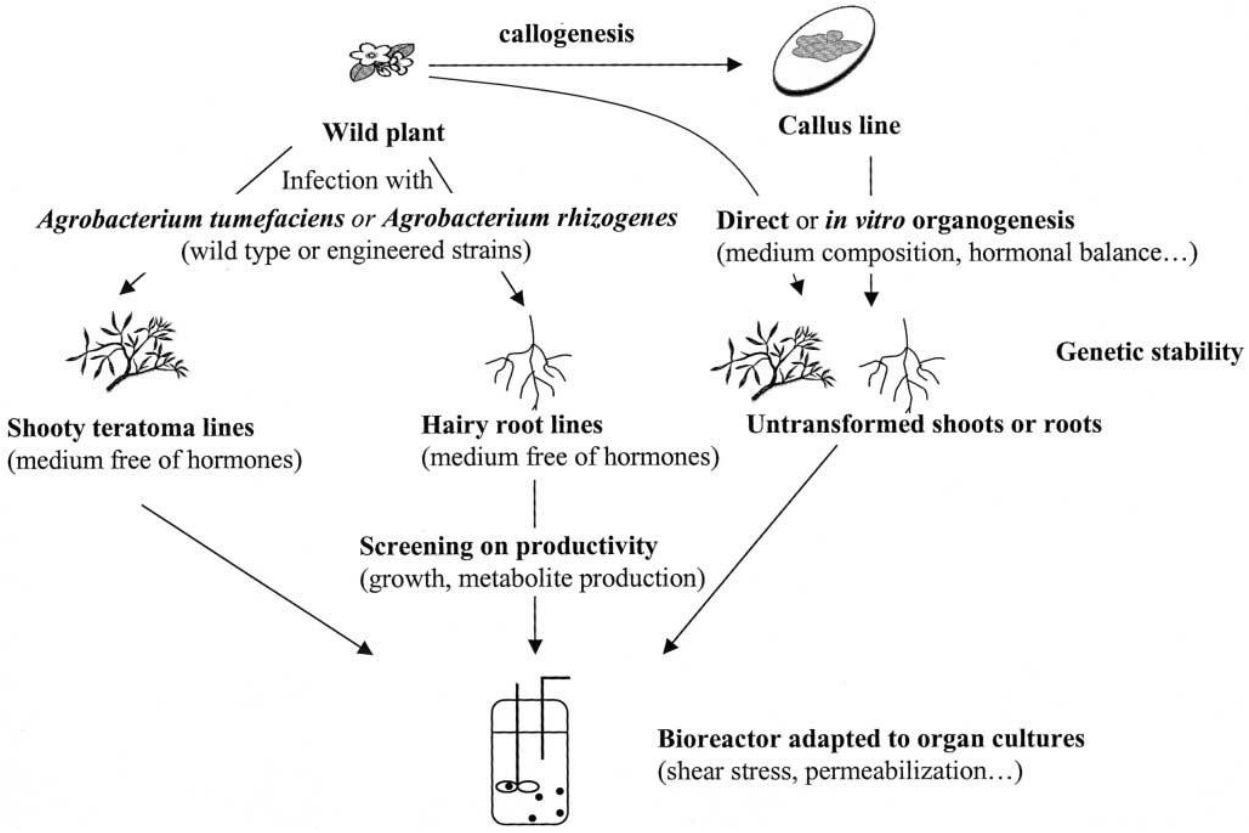


Figure 1. 2 Guidelines for the production of secondary metabolites from plant organ cultures. (Bourgaud et al., 2001).

Plant organs: hairy roots. It has been found that, in differentiated tissues, secondary metabolites accumulation is usually higher than callus and cells without differentiation (Gaosheng & Jingming, 2012). Therefore, the organized cultures, and especially root cultures, can make a significant contribution in the production of secondary metabolites. The hairy root system based on infection and transformation with *rol* genes of *Agrobacterium rhizogenes* has become popular in the last two decades as a method of producing secondary metabolites synthesized in plant roots (Pistelli et al., 2012). For many medicinal herbs, roots are the drogue used for extraction, such as *Panax giseng*, *Panax notogiseng*, *Coptischinensis*, *S. miltiorrhiza.*

Therefore, hairy root can maintain the specificity of the tissue for the synthesis of the bioactive compounds (Gaosheng & Jingming, 2012).

Some researches papers show that the integration of *A. rhizogenes* DNA can lead to modifications of secondary metabolite profile and hairy roots may produce new compounds that normally are not present in intact plants (Łukasz Kuźma et al., 2012; Sheludko et al., 2002). However, normal cell culture can also lead new compounds that have not been isolated from whole plants (Qiao et al., 2012; D.-W. Zhang et al., 2013).

Plant organs: shoots. It is possible to cultivate plant aerial parts (shoots). Maintaining the integrity of the tissue organization, shoots exhibit some similar properties to hairy roots, namely genetic stability and complete capacities for secondary metabolite production, and also the possibility of gaining a link between growth and the production of secondary compounds (Bourgaud et al., 2001). However, as some synthetic pathways are specifically located in root or shoot, there are some differences in the metabolic profiles of the two organs even if they come from the same genotype (Subroto et al., 1996). The major constraint for shoot is the necessity to expose plant material to light for at least 12 hours a day which can be a problem in case of culture with large tank reactors made from steel (Bourgaud et al., 2001).

1.1.5. Molecular pharming: plant tissues for Biopharmaceutical production

In the past 20 years, plant cells have been demonstrated to be an attractive heterologous expression host (using whole plants and *in vitro* plant cell cultures) for foreign protein production (Huang et al., 2012), offering to the biopharmaceutical industry practical and safe advantages over traditional approaches (Moustafa et al., 2016). In the past, commercial production of therapeutic proteins was produced by transformation of bacteria and mammalian cells. e.g. the production of human insulin in *Escherichia coli* (Goeddel et al., 1979). Bacteria are very easy to grow showing fast exploitation but plants are one of the most powerful alternatives for the bioproduction platforms because of their economic and safety advantages (Korde et al., 2016). This was established in recent years through the development of molecular pharming applied to plants and the discovery of ever more efficient techniques for plant transformation, which allowed us to use plants not only for the production of plant-

derived molecules, but also as a production system of exogenous molecules of industrial interest. Plant transformation allows the further use of plants for the production of engineered compounds, such as vaccines and multiple pharmaceuticals (Espinosa-Leal et al., 2018).

In 2012, the first biopharmaceutical Elelyso® (taliglucerase alfa), an enzyme produced in genetically modified carrot cells, was placed on the market for the treatment of type 1 Gaucher disease. This is the first herbal drug approved by (FDA) and, for the Israeli company Protalix Bio Therapeutics of Carmiel, it is the first product made with their ProCellEx protein expression system. To date there are some biopharmaceuticals produced by different plants and for various diseases that are undergoing clinical experiments (**Table 1.1**).

Table 1. 1 Biopharmaceuticals undergoing clinical trials (U.S. National Institutes of Health Clinical Trial) Home Page. Available online: https://clinicaltrials.gov (accessed on 11 June 2019).

Product	Host	Application	Clinical trial	Sponsor
Taliglucerase alfa	Carrot cell culture	Gaucher disease	**FDA approved (2012)**	Pfizer
ZMApp	Tobacco	Ebola virus	Phase 2-3	Map biopharmaceutical
PRX-102	Tobacco cell culture	Fabry disease	Phase 3	Protalix
Moss-aGAL	Moss	Fabry disease	Phase 2-3	Greenovation Biotech GmbH l
Vaccine Pfs25 VLP	Tobacco	Malaria	Phase 1	Fraunhofer, Center for Molecular Biotechnology
Vaccine Recombinant protective antigen	Tobacco	Anthrax	Phase 1	VaxGen
HAI-05	Tobacco	H5N1 vaccine	Phase 1	Fraunhofer, Center for Molecular Biotechnology
Recombinant human intrinsic factor	Arabidopsis thaliana	Vitamin B12 deficiency	Phase 2	University of Aarhus
H5-VLP + GLA-AF Vaccine	Tobacco	Influenza A subtype H%N! infection	Phase 1	IDRI (Infectious Disease Research Institute)
P2G12 Antibody	Tobacco	HIV	Phase 1	St George's, University of London

1.1.6. Bioreactors for biomass scale-up at industrial level

Large-scale plant tissue culture is possible using bioreactors, they offer a controlled supply of biochemical's independent of plant availability (Sajc et al., 2000); they represent the final step in the development of process for producing metabolite from plant *in vitro* culture (Bourgaud

et al., 2001). Bioreactors are self-contained systems with a sterile environment designed to optimize and monitor as well as provide homogenous culture environment conditions such as pH, dissolved gases, aeration, and temperature along with in/outflow channels of liquid and air for mass propagation of cells, tissues, somatic embryos or organogenic propagules (Stiles et al., 2013). The goal in bioreactor design and operation is to provide a prolonged and sterile culture environment with efficient mixing and oxygen exchange in an effort to optimize cell growth and achieve high productivity (Su et al., 2007). However there are different factors affecting the synthesis of secondary metabolites in bioreactors: the gaseous atmosphere, oxygen supply and CO_2 exchange, pH, minerals, carbohydrates, growth regulators, the liquid medium rheology and cell density (Ruffoni et al., 2010). Cell suspension and hairy roots cultures require specific bioreactor conditions according to the sensitiveness to shear stress, which is the main responsible for extensive cell death.

Many studies describe alternative agitation processes that tend to lower this lyses such as air-lift or bubble reactors instead of traditional propeller helixes (Jolicoeur et al., 1992).

Bioreactors for cell culture

The problems limiting the commercial exploitation that have been reported about the use of cells for biomass production are related mainly to the low and unstable cell productivity, slow growth, genetic instability, and an inability to maintain photoautotrophic growth (Paek et al., 2005); the difficulties encountered to set up an efficient plant cell culture consider a larger cell size respect to the bacteria and a more complex morphology. Plant cells have a tendency for aggregation, time-dependent rheological behavior, foaming and wall growth, shear sensitivity, and relatively low growth and oxygen uptake rate (Xu et al., 2011). The design of bioreactors is critical for a successful development of a good large-scale production process (Spier et al., 2011). It is possible to classify bioreactors by agitation methods and vessel construction:

- Mechanically agitated (stirred tank bioreactor, rotating drum bioreactor, spin filter bioreactor).
- Pneumatically agitated and non-agitated bioreactors (simple aeration bioreactor, bubble column bioreactor, air-lift bioreactor, balloon type bubble bioreactor-BTBB).

The first commercial application of large scale cultivation of plant cells was carried out in stirred tank reactors of 200 liter and 750 liter capacities to produce shikonin by cell culture of *Lithospermum erythrorhizon* (Payne et al., 1987).

Depending on the feeding protocol (Ruffoni et al., 2010) classified bioreactors in:

- Batch culture (*close system*): inoculation of organism in a fixed volume of culture medium. The organisms perform a sigmoid growth curve reaching a plateau at the senescence.
- Fed batch culture: the culture medium is gradually added to the bioreactor with a consequent rise of plant biomass (linear relationship).
- Continuous culture during the exponential phase a volume of fresh medium is added and at the same time and equal volume of cell culture is discarded.

Bioreactors for hairy root culture:

One of the huge problems occurring during the growth biomass of hairy roots is the impossibility to contain homogeneous sample tissue due to the complex morphology, non-uniform, and the grow in approximately spherical clumps in liquid medium (Bourgaud et al., 2001; Patra et al., 2016; Ruffoni et al., 2010; Stiles & Liu, 2013). The hairy roots form an interlocked network which limits the transfer of nutrient and oxygen (Shiao et al., 2000; Williams et al., 2000) forming non-homogenous culture environment thus giving rise to a pack of senescent tissues (Khan et al., 2018). For large-scale culture of hairy roots, different bioreactor configurations have been used.

Y. J. Kim et al. (2002), classified the bioreactors for hairy roots into three main types:

- Liquid phase reactors: in these reactors, hairy root biomass is completely submerged in the liquid media and oxygen is usually supplied by bubbling air through the culture medium. However, the mass transfer of gaseous media is rate limiting. We can list as example: stirred tank reactors, bubble column reactors, air lift reactor, submerged convective flow reactors etc…
- Gas phase reactors: in these bioreactors, hairy root biomass is not submerged in liquid and the roots are exposed to air and mixture of air and liquid media. Gas phase reactors include trickle bed reactor, droplet phase reactor, nutrient mist reactor etc…

- Hybrid reactors (a combination of liquid and gas phase): the concept is the combination of liquid-phase and gas-phase reactor system for the most effective hairy root cultivation. A combination of bubble column and trickle bed reactor was suggested by (Flores et al., 1992) as the best compromise.

Simple bioreactors: Temporary Immersion Systems (TIS)

The temporary immersion systems are simple and small bioreactors useful mainly for research purpose that combine a dry culture phase and immersion in the culture medium. One of most common TIS vessel is the RITA® (Recipient à Immersion Temporaire Automatique) system patented by CIRAD, France, distributed by VITROPIC, France and it has been developed for plant micropropagation; the scheme of functioning is showed in **Fig**. **1.3**.

The module consists of a single polypropylene vessel (500 mL) with two compartments, separated by an installed tray with a mesh support and a plastic pipe, mounted to its center. TIS was developed to allow cycling of the culture medium by using air pressure or pump to push the medium from one vessel to the other to immerse the plant tissues and using gravity to withdraw the medium when pump is turned off, thus the plant tissues or immobilized plant cell are exposed to the medium intermittently rather than continuously (Huang & McDonald, 2012). The advantages of the RITA® vessels are the simple and reliable operation, the compact space in the growth chamber, and the support of right relative humidity level with full separation of the propagules and liquid medium (V. Georgiev et al., 2014), so reducing and solve an important problem that occurs in shoot propagation named hyperhydration that damage seriously the plant material. RITA® also presents the advantage of low consumable and labor costs (Mc Alister et al., 2005). This system has been used to produce secondary metabolites in *in vitro* cultivation of plant tissue or organ cultures in liquid medium. e.g. the production of galanthamine on shoots of *Leucojum aestivum* (Ivanov et al., 2011), the production of secoiridoid glycosides on *Centaurium maritimum* (L.) hairy root culture (Mišić et al., 2013) and the production of betalains by *Beta vulgaris* L. hairy root culture (Pavlov et al., 2006).

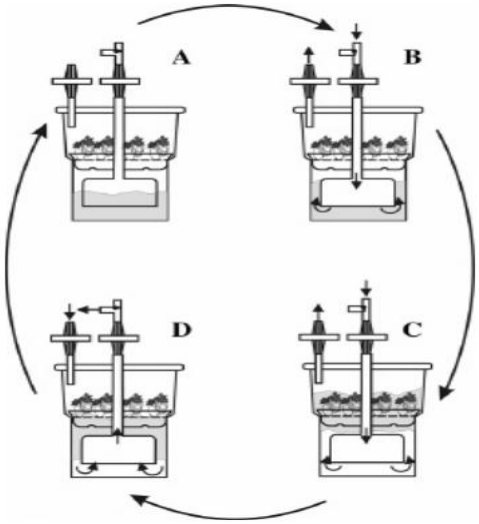


Figure 1. 3 Technological design and operational principle of RITA® system: (A) period of exposure; (B) Dislocation of liquid medium. Air pressure is applied to the bottom compartment through the central pipe. The liquid medium is moving to the upper compartment; (C) period of immersion; (D) draining out the nutrient medium. The air flow is stopped and the medium flows back to the bottom compartment due to gravity. *Source:* (V. Georgiev et al., 2014).

1.2. Plant genetic transformation

Transformation is a molecular biology method that alters the genome of cells through incorporation of desired foreign genetic material (Dhatwalia et al., 2017). There are two types of gene transfer methods into plants, direct and indirect. The last (indirect) is based on the use of soil-borne *Agrobacterium tumefaciens* and *A. rhizogenes* as vectors; this is also called natural genetic engineering and the first(direct) is based on the use of physical, electrical or chemical treatments to introduce desired DNA into the plant cell (Vinod et al., 2017).

Direct transfer of foreign DNA into the plant

The polar property of gene does not get transferred directly to the plant cell due to the hydrophobic and hydrophilic nature of plasma membrane. To facilitate this transfer, many physical and mechanical methods using the selective chemical environment for uptake of foreign gene into recipient cells were developed. Among these methods we can list: Particle

bombardment, microinjection, electroporation, protoplast transformation, sonoporation, etc…(Choudhary et al., 2017).

Indirect transfer of foreign DNA in to the plant

For indirect gene transformation method, schematized in **Fig. 1.4**, two bacterial species *Agrobacterium rhizogenes* and *A. tumefaciens* have been discovered to be able to insert in a stable way, after infection, fractions of their DNA into the plant cells (Choudhary et al., 2017).

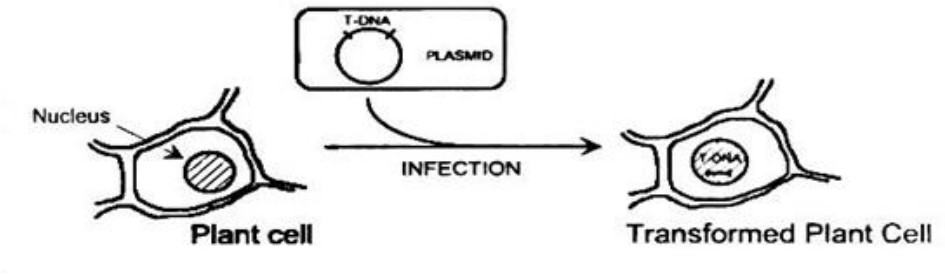


Figure 1. 4 Insertion of T-DNA from *Agrobacterium sp* to plant cell.

1.2.1. *Agrobacterium*-mediated genetic transformation

The processing mechanism needed to T-DNA production and transfer into plant host cell comprises proteins that are encoded by a set of bacterial chromosomal and Ti (tumor inducing) or Ri (root inducing) plasmid genes known as *vir* genes (virulence genes) located on the virulent region (Pistelli et al., 2010; Stachel et al., 1986; Tzfira et al., 2006).

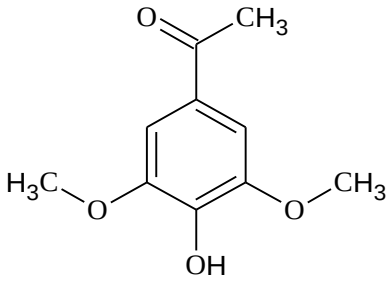
In addition, various host proteins have been reported to participate in the Agrobacterium-mediated genetic transformation process (Gelvin, 2003; Tzfira et al., 2002), mostly during the later stages of the process (i.e. T-DNA intracellular transport, nuclear import and integration).

The 30 kb *vir* region is organized into six complementation groups that are either absolutely essential for (*virA, virB, virD,* and *virG*) or that enhance the efficiency of (*virC* and *virE*) plant cell transformation (Stachel et al., 1986). Those *vir* genes directly involved in T-DNA processing and transfer, are tightly regulated so that expression occurs only in the presence of wounded plant cells, the targets of infection (Zupan et al., 1995).

In the vegetative bacterium, only *virA* and *virG* genes are significantly expressed; however, when *Agrobacterium* is co-cultivated with plant cell the expression of other *vir* genes is induced to high levels.

The mechanism of transfer of T-DNA can be explained as follow (Gelvin, 2017; Pistelli et al., 2010; Zupan & Zambryski, 1995) and schematically explained on (**Fig. 1.5**):

- Wounding of plant tissue induces the production of numerous defense compounds, including some phenolics that can, along with neutral and acidic sugars, trigger a sensory response system (Gelvin, 2017). However, acetosyringone an activator of the *vir* genes of *Agrobacterium* can be used to stimulate the hairy root induction (Stachel et al., 1985).



Acetosyringone

Ozyigit et al. (2013), declared that phenolic compounds released from wounded sides cause attraction of *A. rhizogenes* which moves toward the wounded sites by chemotaxis and infect plant cells.

- VirA detects these small phenolic compounds released by wounded plants resulting in autophosphorylation. VirA phosphorylation of VirG then leads to activation of *vir* gene transcription (Zupan & Zambryski, 1995).
- The expression of the *virD* locus caused double-stranded cleavage of the T-DNA at or near the borders and generated single-stranded T-DNA molecules (Jayaswal et al., 1987). VirDl and VirD2 are essential for this process; VirD1 is a helicase, and VirD2 is a strand-specific endonuclease which cuts the T-DNA border repeats and remains attach covalently to one end initiates packaging of one strand into a linear complex (with the 5' end of the T-strand) forming virD2-T-strain (immature T-complex).

- virD2-T-strain and several vir proteins (VirE2, VirE3, VirF and VirD5) are exported through membranes and cells walls to the host cell cytoplasm by T4SS, encoded by the *virB* operon and the *virD4* gene (Pistelli et al., 2010). It was showed that virB was necessary for tumorigenesis but was not required for T-strand or T-complex formation (Stachel et al., 1986)
- Once inside the host cell virD2-T-strain is coated tightly with VirE2 protein (encoded by the *virE* locus) leading to mature T-complex (T-strand along with VirD2 and VirE2) (Pistelli et al., 2010).
- Mature T-complex is then imported into the host nucleus with the assistance of several host and bacterial factors, uncoated by targeted proteolysis and randomly integrated by an undetermined mechanism into the plant genome.

-

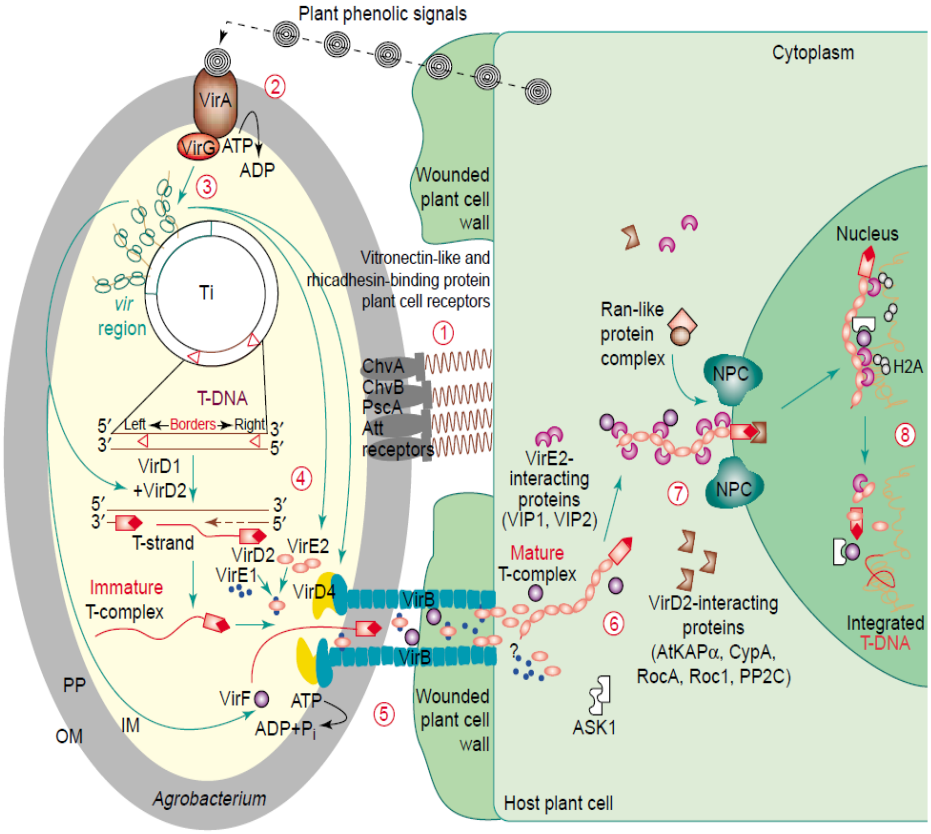


Figure 1. 5 Model of molecular interactions during *Agrobacterium* mediated genetic transformation of plant cells. The transformation process comprises eight distinct steps: (1) *Agrobacterium* recognition of and attachment to the host cell, mediated by *Agrobacterium* chromosome-encoded proteins and specific host receptors; (2) sensing of specific plant signals by the *Agrobacterium* two-component (VirA–VirG) signal-transduction system machinery; (3) VirG-mediated signal transduction and *vir* gene activation; (4) generation of mobile copy of T-DNA, the T-strand; (5) generation of the VirB–VirD4 transporter complex, and transport of T-strands and Vir proteins into the host cell cytoplasm; (6) formation of the mature T-complex; (7) T-complex nuclear import facilitated by the AtKAPα, VIP1 and Ran proteins of the host cell; (8) intra-nuclear transport of the T-complex to the host chromosome, and T-DNA integration into the host cell genome mediated by VirD2 and/or VirE2 and by host factors. Abbreviations: IM, bacterial inner membrane; NPC, nuclear pore complex; OM, bacterial outer membrane; PP, bacterial periplasm. Source: (Sheng et al., 1996; Tzfira & Citovsky, 2006).

1.2.2. *Agrobacterium rhizogenes* and Ri plasmid

1.2.2.1. Agrobacterium rhizogenes

Agrobacterium rhizogenes is a Gram negative soil-borne bacterium of the family of *Rhizobiaceae* that causes the hairy roots disease by infecting wonder high plant (Christey et al., 2005), principally dicotiledons species. It was identified in 1930 by Riker (Riker et al., 1930). *A. rhizogenes* present a rod-shaped non-spore forming (0.6–1 µm by 1.5–3.0 µm in size) and is motile by means of one to six peritrichous flagella (C. Giri et al., 2007). It is a close relative of the better known *A. tumefaciens*, which is the best-characterized species among the genus *Agrobacterium* (Özyiğit, 2012).

1.2.2.2. Ri plasmid

All *A. rhizogenes* strains are characterized by the presence of a large root inducing (Ri) plasmid (**Fig. 1.6**) containing a highly conserved "core" DNA region required for hairy root formation (Veena et al., 2007). The pathogen infect the plant cell by transfers a DNA (T-DNA) segment (T-DNA region bounded by 25 bp direct oligonucleotide repeats) which carries genes (*rol* genes) from its (Ri) plasmid into the genome of plant (Pistelli et al., 2010). This T-DNA also carries a set of oncogenes and opine-catabolism genes, whose expression in plant cell leads to neoplastic growth of the transformed tissue and opines production. These compounds are used by bacteria as the sole carbon and nitrogen sources for further growth (Srivastava et al., 2007; Zupan & Zambryski, 1995). Expression of Ri T-DNA leads to abundant proliferation of roots at the site of inoculation. Such "hairy roots" can readily be grown *in vitro* as root cultures (Zambryski et al., 1989). The production of the hairy root phenotype involves the integration of *rol* genes from the Ri plasmid.

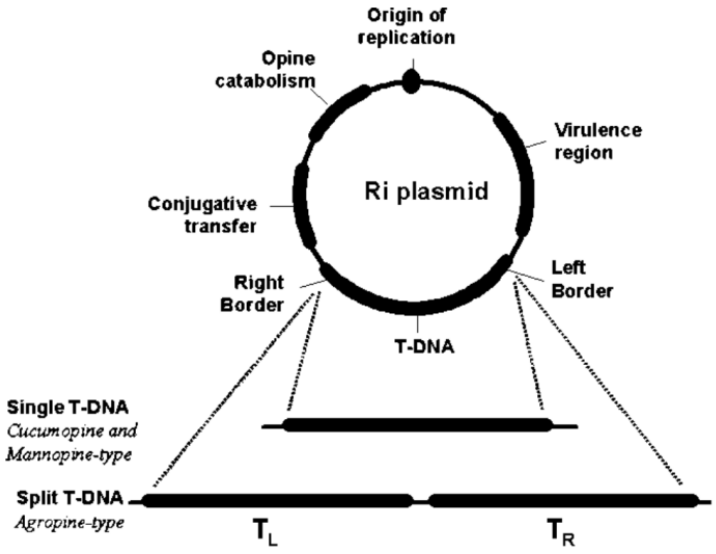


Figure 1. 6 Simplified structure of *A. rhizogenes* Ri plasmids.

e.g. *Agrobacterium rhizogenes* strain 15834, which incites hairy root disease in plants, harbors three large plasmids: pAr15834a (107 x 10' daltons), pAr15834b (154 x 10' daltons), and pArl5834c (258 x 10" daltons).

The development of an efficient protocol for successful hairy root induction by *Agrobacterium rhizogenes* is the key step toward an *in vitro* culturing method of hairy root for the mass production of secondary metabolites.

1.2.2.3. Hairy roots: characteristics, advantages and disadvantages

Using of hairy roots in plant biotechnologies started during 1930s (White, 1934). During 1980s and in the beginning of 1990s this type *in vitro* cultures attracted attention of the researchers to investigate them as an experimental matrix for production of valuable secondary metabolites. The **Table 1.2** illustrates the advantage and disadvantage of hairy roots culture.

Table 1. 2 Advantages and disadvantages of hairy roots for producing plant secondary metabolites (Kümmritz et al., 2017).

Advantage	Disadvantage
Genetic and biochemical stability	Availability in their genome of t-DNA of Agrobacterium rhizogenes
Growing in media free of growth regulators	Mainly dicotyledonous plants could be transformed with Agrobacterium rhizogenes
Synthesized metabolites are with comparable yields to the intact plants	Produce mainly metabolites that are synthesized in the roots of corresponded intact plants
Stable growth characteristics at the cultivation in bioreactors	Bioreactor design should be adopted to the morphological peculiarities of the hairy roots
Missing of geotropism	
Possibility of manipulation of biosynthetic pathways	
Fast growth and short doubling time comparable to plant suspension cultures	

As of today, the hairy root culture found large application into different fields as illustrated on **Fig. 1.7**.

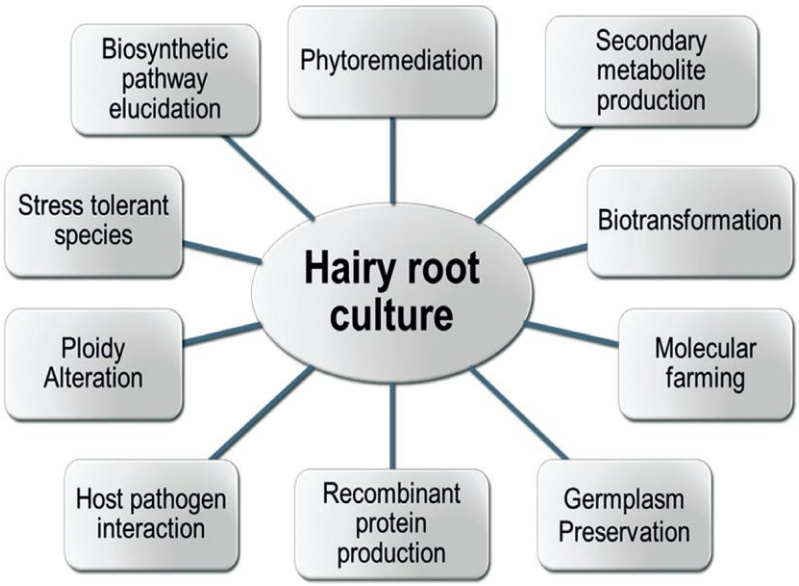


Figure 1. 7 Application of hairy root culture

1.3. *Salvia* species

Salvia is the largest plants genus of mint family (Will et al., 2015), lamiaceae, with nearly 1000 species of shrubs, herbaceous, perennials and annuals (Walker et al., 2004). Among the 57 largest genera (2002), *Salvia* is at the 20th place and is considered a mark of relatively high world and regional diversity (Frodin, 2004). It is widely distributed in the temperate, subtropical, and tropical regions (Sharifi-Rad et al., 2018). The *Salvia* species undergone marked species radiations in three regions of the world: Central and South America (500 spp.), central Asia/ Mediterranean (250 spp.), and eastern Asia (90 spp.) as showed in **Fig. 1.8** (Walker et al., 2004). Will et al. (2017), showed that *Salvia* species is polyphyletic (descent from more than one ancestral group of plants) with four distinct evolutionary lineages (Clade I-IV), and these clades strongly reflect the geographical distribution, i.e., Clade IV (East Asia), Clade III (Southwest Asia to Northern Africa), and Clade II (America). The origin of Salvia (Clade I) is most likely Southwest Asia.

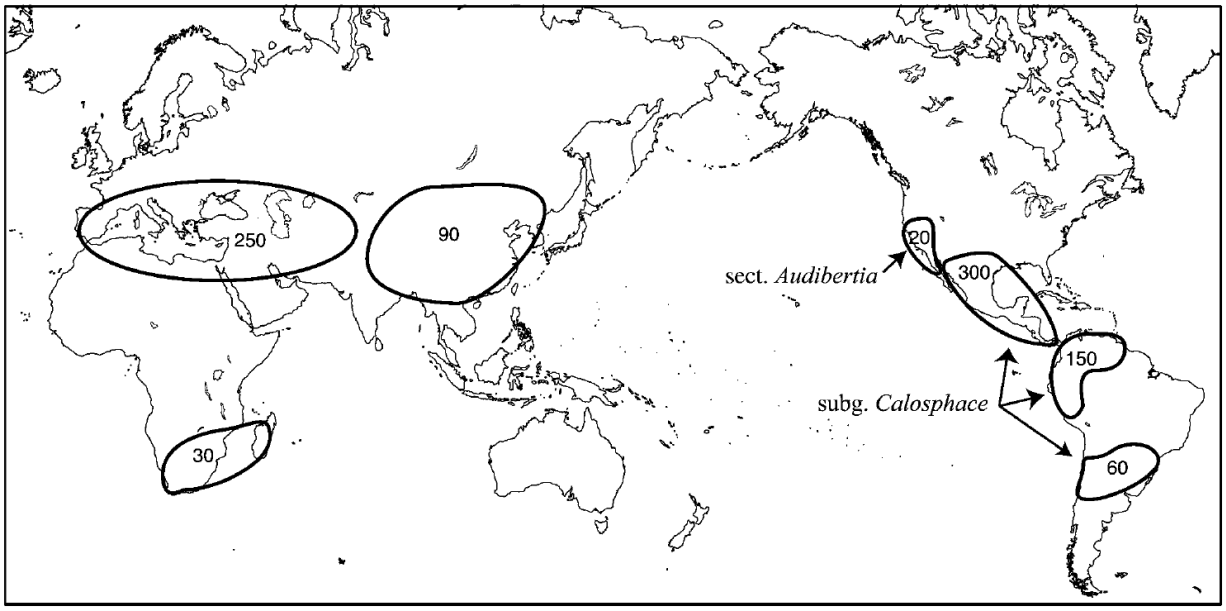


Figure 1. 8 Centers of diversity for the genus *Salvia*. Approximate numbers of species in each region are listed inside each area. (Walker et al., 2004).

Many *Salvia* species are cultivated for their aroma and secondary metabolites, they are used for food additives and for the production of essential oils, pharmaceuticals, colorants, dyes, cosmetics, and biocides (Lubbe et al., 2011).

1.3.1. Ethnopharmacognosy of *Salvia* species

For a long time, *Salvia* species are known for a wide variety of medicinal used in folk medicine for the relief of pain, protecting the body against oxidative stress, free radical damages, angiogenesis, inflammation, bacterial and virus infection, etc… (Hamidpour et al., 2014). In fact, the name *Salvia* is derived from the Latin word salvare, which refers to its healing qualities (Llurba-Montesino et al., 2018). In China, about 40 *Salvia* species have been used as medicinal plants for treatment of various diseases, specifically hepatic and renal diseases and those of the cardiovascular and immune systems (Jianping et al., 2018). The interest on *Salvia* plants for food and pharmaceutical applications is wide and growing through reporting their biological properties, including antioxidant, antimicrobial, anti-alzheimer, hypotensive, anti-hyperglycemia, antihyperlipidemia, anti-cancer, and skin curative agents (Sharifi-Rad et al., 2018). Finally, medicinal applications of *Salvia* are attributed to different phytochemicals present in various species. Based on their widespread ethno-application, some *Salvia* species (*S. sclarea, S. officinalis, S. fruticosa* syn. triloba and *S. lavandulaefolia*) are recognized as safe in the USA (FDA, 2012) and their medicinal products are officially presented in the European Pharmacopoeia VI edition (A Marchev, 2014).

1.3.2. Phytochemical composition of *Salvia* species.

The ethnopharmacological value of *Salvia* has led researchers to focus on the metabolite content of its different parts (roots and aerial part). The medicinal applications of *Salvia* are attributed to different phytochemicals present in various species. From a phytochemical point of view, Salvia spp. has a complex chemical composition being terpenes and polyphenols their main compounds (Porres-Martínez et al., 2017; Topçu, 2006). Until the beginning of 2011, a total of 773 compounds were isolated from 134 Salvia species and were classified into seven groups: sesquiterpenoids, diterpenoids, sesterterpenoids, triterpenoids, steroids, polyphenols,

and others. More than 80% are terpenoids, especially diterpenoids; relatively, sequiterpenoids and triterpenoids are rare in the *Salvia* species. According to their structure, diterpenoids are further classified into five subgroups: abietane diterpenoids, clerodane diterpenoids, pimarane diterpenoids, labdane diterpenoids, and other diterpenoids (Y.-B. Wu et al., 2012).

1.3.3. *Salvia* and biotechnologies for industrial application

The economic importance of this genus has increased in recent years due to evidence that some of its secondary metabolites have valuable pharmaceutical and nutraceutical properties. The bioactivity of sage extracts is mainly due to their content of terpenes and polyphenols. The increasing demand for sage products combined with environmental, ecological and climatic limitations on the production of sage metabolites from field-grown plants have led to extensive investigations into biotechnological approaches for the production of *Salvia* phytochemicals (Andrey Marchev et al., 2014).

The growing demand has led to overexploitation of natural habitats, and in the last few years, many wild growing Salvia species have shrunk or fallen under threat. Obviously, to deal with that problem and to prevent ecological crisis, there is an urgent need for alternative, renewable source of Salvia biomass. Plant *in vitro* culture technology is a powerful method for continuous production of plant secondary metabolites under controlled conditions, recently adapted to various Salvia species. *Salvia in vitro* systems are harmless to natural plant populations and can be grown independently of environmental factors, geographical latitude, climatic change, and seasonal variations. Several bioactive compounds from rare and endangered *Salvia* plants can be produced by employing different plants *in vitro* systems (V. Georgiev et al., 2017) such as shoots, cell culture, hairy roots, etc… (Bisio et al., 2016; Izabela Grzegorczyk et al., 2005).
Biotechnological techniques significantly facilitate plant propagation and production of some important bioactive compounds from the genus *Salvia* (Kintzios et al., 1999). Different methodologies of *in vitro* plant tissues and cell cultures may represent an alternative strategy for the production of highly valuable plant metabolites and could be even adopted by pharmaceutical industries to develop new drugs and formulations (Marco Savona et al., 2017). However, the achievements in development of large-scale biotechnology, based on Salvia *in*

vitro systems, are still in early stage and many points still have to be addressed before the commercialization to take place (V. Georgiev & Pavlov, 2017).

Different methodologies of *in vitro* culture can be employed to produce biomass of medicinal plant regardless the conservation of both phenotype and genotype of initial plant. The first successful hairy root transformation of tissues from a plant of the genus Salvia to produce bioactive compound was carried out by Zhi et al. (1993), on *S. miltiorrhiza*. Few years later, the transformation is widely on others *Salvia* species **Table 1.3.**

However, using the transformation approach to produce secondary metabolite can leads to obtain the formation of new compound non-present in the root of whole plant.

Table 1. 3 List of *Salvia* species transformed by *A. rhizogenes* and secondary metabolites produce.

***Salvia* species**	***A. rhizogenes* strains**	**Secondary metabolites**	**References**
miltiorrhiza	LBA 9402, ATCC 15834, TR 105, R1601 and A4 1027.	Diterpenoid (tanshinones)	(Zhi & Alfermann, 1993)
cinnabarina	ATCC 15834	Ursolic acid,	(M Savona et al., 2003)
broussonetii	ATCC 15834	New diterpenes	(Fraga et al., 2005)
involucrata	R1601	Flavonoids (apigenin)	(Izabela Grzegorczyk et al., 2006)
***officinalis* L.**	ATCC 15834 and A4	Rosmarinic acid	(Izabela Grzegorczyk et al., 2006)
***sclarea* L.**	LBA 9402 ATCC 15834	Diterpenoids Phenolic acids and flavonoids	(Różalski et al., 2007) (Andrey Marchev et al., 2011)
***austriaca* Jacq.**	A4	Diterpenoid	(Ł Kuźma et al., 2011)
***wagneriana* Polak**	ATCC 15834	Rosmarinic acid	(Ruffoni et al., 2016)
viridis	A4	Polyphenolic compounds	(Grzegorczyk-Karolak et al., 2018)

Plant cell culture (normal or transformed cells) technology offers an alternative means of producing these secondary metabolites. The first study on untransformed cell suspension cultures and immobilized cells of *S*. *miltiorrhiza* have been carried out and reviewed by H. Miyasaka et al. (1989). Few years later, H. Chen et al. (1997) established transformed cell cultures of *S*. *miltiorrhiza* by infecting sterile plantlets with *Agrobacterium tumefaciens* strain C58. Transformed cell cultures may have several advantages over normal cell cultures including

no requirement for exogenous phytohormones, fast growth rates and the introduction of foreign genetic materials into the plant cells which may favor the formation of secondary metabolites (Towers et al., 1993). However, from genus *Salvia,* only *Salvia officinalis, fruticosa* and *miltiorrhiza* were investigated through cell culture to produce secondary metabolite (**Table.1.4**).

Table 1. 4 Cell culture established from *Salvia* species to produce secondary metabolites.

***Salvia* species**	**Type of cell culture**	**Secondary metabolites**	**References**
officinalis	Cell culture	Rosmarinic acid	(Hippolyte et al., 1992)
fruticosa	Cell culture	Rosmarinic acid	(Karam et al., 2003)
miltiorrhiza	Ti/transformed cell	Tanshinones	(H. Chen et al., 1997)

1.4. Diterpene: Biosynthetic pathway, *in vitro* production and biological activity

1.4.1. Biosynthesis pathway of abietane diterpenes in *Salvia* specie

The development of successful metabolic engineering approaches for diterpenes production requires an in-depth understanding of the pathways that contribute to their biosynthesis.

The biosynthesis of majority secondary metabolites involves multistep reactions and many enzymes. Biosynthesis of terpenoids so diterpenoids, can be divided into four stages (B. M. Lange et al., 2013) (**Fig 1.9**). At the first stage, the universal isoprene precursor isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate can be synthesized by two distinct isoprenoid biosynthesis pathways, the mevalonate (MVA) pathway in the cytosol and/or the 2-*C*-methyl-D-erythritol-4-phosphate (MEP) pathway in the plastids (Bouwmeester, 2006; Liao et al., 2009; Rodríguez-Concepción et al., 2015; Vranová et al., 2013). At the second stage, geranylgeranyl diphosphate synthase (GGPPS) catalyzes the synthesis of the intermediate diphosphate precursor geranylgeranyl diphosphate (C_{20}) from DMAPP and three molecules of IPP (Kai et al., 2010; Liao et al., 2009). GGPPS isoforms have been reported to occur in plastids, the endoplasmic reticulum (ER) and mitochondria (Liao et al., 2009; Okada et al., 2000). At the third stage, diverse diterpenoid are synthesized through the catalysis of terpene synthases/cylases including copalyl diphosphate synthase (CPS), kaurene synthase-like (KSL), miltiradiene oxidase CYP76AH1, and other enzymes (J. Guo et al., 2013; Y. Ma et al., 2012). Božić et al. (2015), described the isolation and characterization of the genes, namely SfCPS, SfKSL and SfFS, coding for enzymes involved in the biosynthesis of carnosic acid precursors in the young trichomes of *S. fruticosa*. At the last stage, skeletons are further functionalized, through redox, conjugation and other modifying reactions, to yield a wide range of end products. Cytochrome P450 monooxygenases frequently catalyze secondary transformations of the newly formed polycyclic diterpenes (Božić et al., 2015). B Markus Lange et al. (2003), reported that it is thought that the MEP pathway is prevalent in the biosynthesis of mono- and diterpenoids.

The biosynthesis pathway icetexane skeleton (such as demethylfruticuline A and fruticuline A) is not known. However, biosynthetic relationship show that the icetexane skeleton is believed to arise from a rearrangement of the more common abietane skeleton, giving rise to a 6-7-6 tricyclic framework that bears the systematic name 9(10/20)-abeo-abietane (Rodriguez-Hahn et al., 1989). In accordance with this hypothesis, the greater number of icetexane natural products that have been discovered to date have been found in plant species which also produce abietane diterpenoids as secondary metabolites. The icetexane natural products that have been discovered to date vary widely in the degree of oxygenation and oxidation in each ring, leading to a diverse array of structures and biological activities (Simmons et al., 2009). The abietane-type diterpenes, miltiradiene and ferruginol, were recently identified as precursors of tanshinones in *S. miltiorrhiza* (Gao et al., 2009; J. Guo et al., 2013).

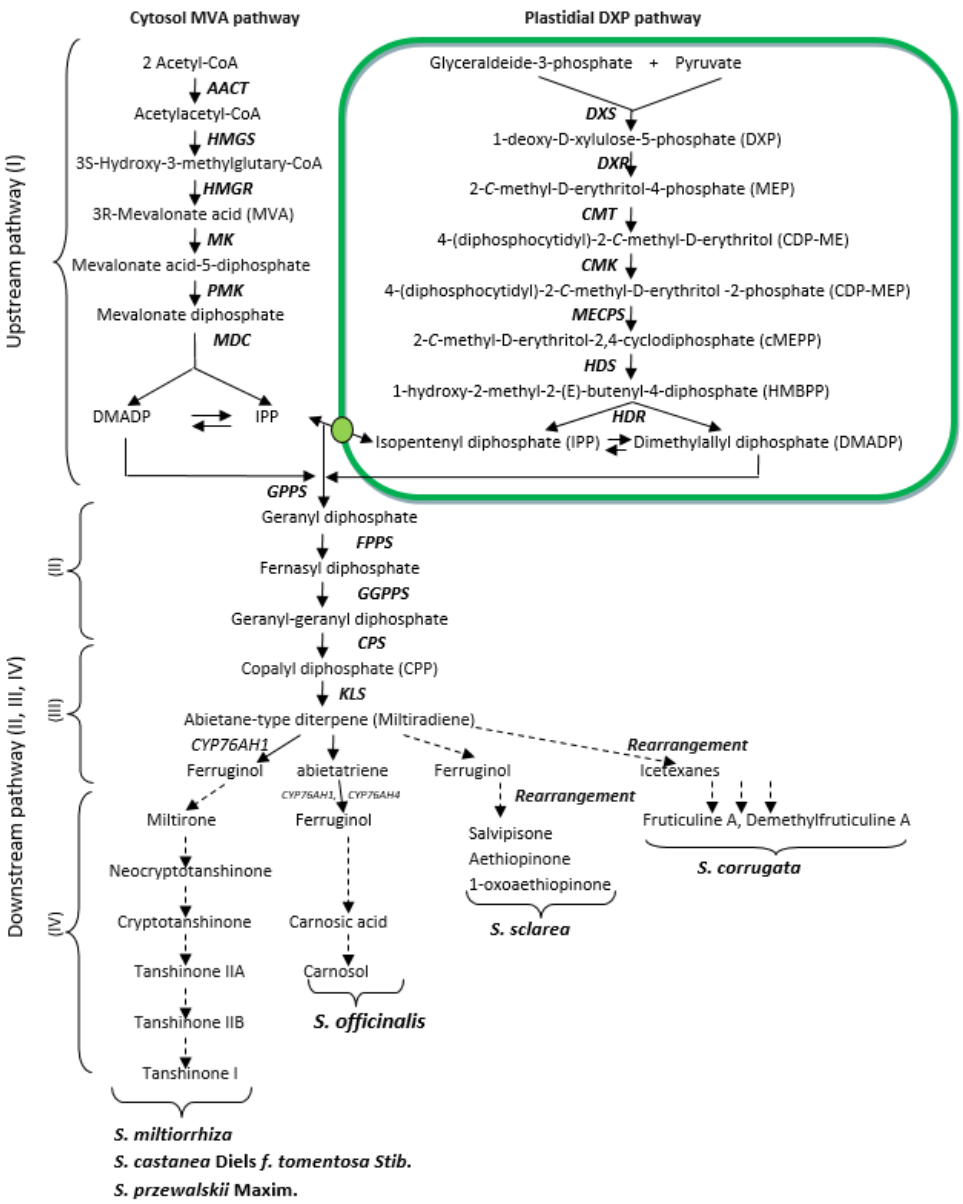


Figure 1. 9 Proposed hypothetical pathway for diterpenes biosynthesis in some *Salvia* species (on findings reported by (Gao et al., 2009; P. Ma et al., 2015; Zi et al., 2013), dashed arrows indicate hypothetical relationships. CMK: 4-(cytidine 5-diphospho)-2-C-methyl-Derythritol kinase, CPS: Copalyl diphosphate synthase, DXR: 1-Deoxy-D-xylulose-5-phosphate reductoisomerase, DXS: 1-Deoxy-D-xylulose-5-phosphate synthase, FPPS: Farnesyl diphosphate synthase, FS: ferruginol synthases, GGPPS: Geranylgeranyl diphosphate synthase, GPPS: Geranyl diphosphate synthase, HDR: 1-Hydroxy-2-methyl-2-(E)-butenyl-4- diphosphate reductase, HDS: 1-Hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase, HMGR: 3-Hydroxy-3-methylglutaryl-CoA reductase, HMGS: 3-Hydroxy-3-methylglutaryl-CoA synthase, IPPI: Isopentenyl-diphosphate delta-isomerase, KSL: Kaurene synthase-

like, MCT: 2-C-methyl-D-erythritol-4-phosphate cytidyl transferase, MDC: Mevalonate 5-diphosphate decarboxylase, MECPS: 2-C-methylerythritol 2,4-cyclodiphosphate synthase, MK: Mevalonate kinase, PMK: 5-Phosphomevalonate kinase.

1.4.2. Diterpene*: in vitro* production and biological activity

Diterpenes constitute one of the largest and most diverse classes of plant metabolites with more than 10,000 different structures, and the diterpenoids isolated in the Lamiaceae present about 50 different skeleton (Bisio et al., 2019). The **table 1.5** lists some diterpenes produced by *in vitro* culture of different *Salvia* species.

- **Fruticuline A and demethylfruticuline A**

Fruticuline A is an icetexane diterpene isolated for the first time from *Salvia fruticulosa* Benth. (Rodríguez-Hahn et al., 1989), after in *S. arizonica* Gray (Valant-Vetschera et al., 2003), *S. corrugata* Vah (Bisio et al., 2008) and *S. lachnostachys* (Erbano et al., 2012). With demethylfruticuline A, fruticuline A are the mains diterpenes constituents in the aerial part of *S. corrugata* Vahl (Bisio et al., 2008). As it was earlier described on establishment of *in vitro* culture of *S. corrugata*, the analysis of the regenerated shoots showed the presence of both icetexanes with the yield of fruticuline A higher in the methanolic extract than in those of fresh leaves and fresh shoot tips. However, micropropagated plants contained only fruticuline A, while the callus contained trace amounts of both diterpenes (Bisio et al., 2016). Fruticuline A showed potent antibacterial activity against Gram-positive (Bisio et al., 2008; Schito et al., 2011). Demethylfruticuline A induces anoikis, a type apoptosis induced in mammalian cells through a loss of cell adhesion mediated by CD36 (Monticone et al., 2010) and also causes apoptosis by inducing reactive oxygen species in mitochondria (Monticone et al., 2010).

- **Carnosic acid, carnosol**

Carnosic acid is one of the most studied phenolic diterpene (Božić et al., 2015) due to its of high importance to the food and cosmetic industry, and pharmaceutical applications, for its strong antioxidant, anti-inflammatory and anticancer properties (Bauer et al., 2012; Lin et al., 2018; Pavić et al., 2019; Poeckel et al., 2008). There is a great need to understand the processes

leading to its biosynthesis and how to increase its amount. Carnosic acid and carnosol are labdane-related diterpenoid, isolated in *Salvia aethiopis* (Rodríguez et al., 1984). Carnosic acid is mostly present in the aerial parts of the plant, and its biosynthesis in leaves has been thoroughly studied in *S. fruticosa* and *R. officinalis* (Abreu et al., 2008; Birtić et al., 2015; Brückner et al., 2014). The production of this compound using genetic engineering could be of great use for the pharmaceutical industry. Unluckily, Izabela Grzegorczyk et al. (2006), demonstrated that the root cultures of *S. officinalis* did not produce these diterpenoids with antioxidant activity. This is not surprising because the secondary metabolites are accumulated in aerial chlorophyllous tissues and were found only in trace amounts in roots of intact plants of *S. officinalis* (Izabela Grzegorczyk et al., 2005).

- **Aethiopinone, salvipisone, ferruginol and 1-oxoaethiopinone**

Rearranged abietane diterpene Aethiopinone and salvipisone were extracted for the first time from root of *Salvia aethiopis* and later the abietane-type ferruginol (Boya et al., 1981; Rodríguez et al., 1984; Ulubelen et al., 1999). These compounds are mostly present in root of some *Salvia* species and were also isolated from roots of *Salvia argentea* (Michavila et al., 1986), *S. candidissima* Vahl. (Ulubelen et al., 1992), *S. ceratophylla L*. (Goren et al., 2002) and from the aerial parts of *S. cyanescens* (Gökdil et al., 1997). All these diterpenes with rearranged abietane 1-oxoaethiopinone were isolated from root *S. sclarea* L. (Ulubelen et al., 1997). Analyzing previous studies, only *Salvia sclarea* was investigated in the biotechnology field to produce these abietane diterpenoids through hairy root transformation (Kuzma et al., 2008; Ł. Kuźma et al., 2006; M. Vaccaro et al., 2014). It is known that the genetically modified roots grown *in vitro* are often able to biosynthesize valuable secondary metabolites, usually in higher amounts than intact plants (Ł Kuźma et al., 2017; Subroto et al., 1994). As it was earlier described, *in vitro* hairy root transformation of *S. sclarea* by *Agrobacterium rhizogenes* LBA 9402 strain is able to produce ferruginol, salvipisone, aethiopinone, and 1-oxo-aethiopinone (Ł. Kuźma et al., 2006). However, the biosynthesis mechanism and enzymes involved in the process of production are not known.

These compounds have various biological activities. Amount them, ferruginol exhibited a strong anti-acanthamoeba (Ł. Kuźma et al., 2015) and an *in vitro* antiplasmodial activities (Ebrahimi et al., 2013). Aethiopinone has been shown to have analgesic and anti-inflammatory properties (Hernández-Pérez et al., 1995). In addition, it was proved to be cytotoxic to different solid tumor cell lines, especially with the highest effect on human melanoma A375 cell line (IC_{50} 11.4 µM) (M. Vaccaro et al., 2014). Salvipisone and aethiopinone showed antibacterial activities against selected methicillin-susceptible and methicillin resistant *S.aureus* and *S. epidermidis strains* (Hernandez-Perez et al., 1999; Różalski et al., 2007; Walencka et al., 2007). These two molecules showed relatively high cytotoxicity against HL-60 and NALM-6 leukemia cells (IC50 range 0.6- 7.7 µg/mL which is equal to 2.0 - 24.7 µM), whereas 1-oxoaethiopinone and ferruginol were less active in this regard (Różalski et al., 2006).

- **Tanshinones**

The root of *S. miltiorrhiza* contains hydrophobic bioactive diterpenes, which mainly tanshinones (dihydrotanshinone I, cryptotanshinone, tanshinone I, tanshinone IIA etc.) (H.-B. Li et al., 2001; M.-H. Li et al., 2008; L. Zhou et al., 2005). It is a commonly used traditional Chinese medicine (TCM) for improving body function such as promoting circulation and improving blood flow (L. Zhou et al., 2005). Tanshinones are widely distributed in the genus *Salvia* (M. H. LI et al., 2010). The roots of *S. castanea* Diels *f. tomentosa* Stib and *S. przewalskii* have been used as a substitute for *S. miltiorrhiza* roots; and it has been reported that the main chemical components are namely abietane type diterpene pigments tanshinones (Bo et al., 1991; L. Liu et al., 2018). Tanshinones were also found in root of *Perovskia abrotanoides*, a member of the Lamiaceae family (Sairafianpour et al., 2001), and its amount was increased by biotics and abiotics elicitors (Zaker et al., 2015). Since the 1990s, several researchers have exploited the *in vitro* culture of *S. miltiorrhiza*, including hairy roots and cell culture to produce tanshinones. Few years later, tanshinone I (T-I) and tanshinone IIA (T-IIA) were measured in roots of micropropagated and *in vitro* regenerated plants of *S. przewalskii* (Skała et al., 2005). Tanshinones have received large attention due to their remarkable activities in the clinical treatment of cardiovascular diseases. Nowadays, these compounds have been found to possess

a wide range of pharmacological activities, such as antibacterial, antioxidant, anti-inflammatory, and anti-tumor properties as reported in **Fig. 1.10** (Jiang et al., 2019)

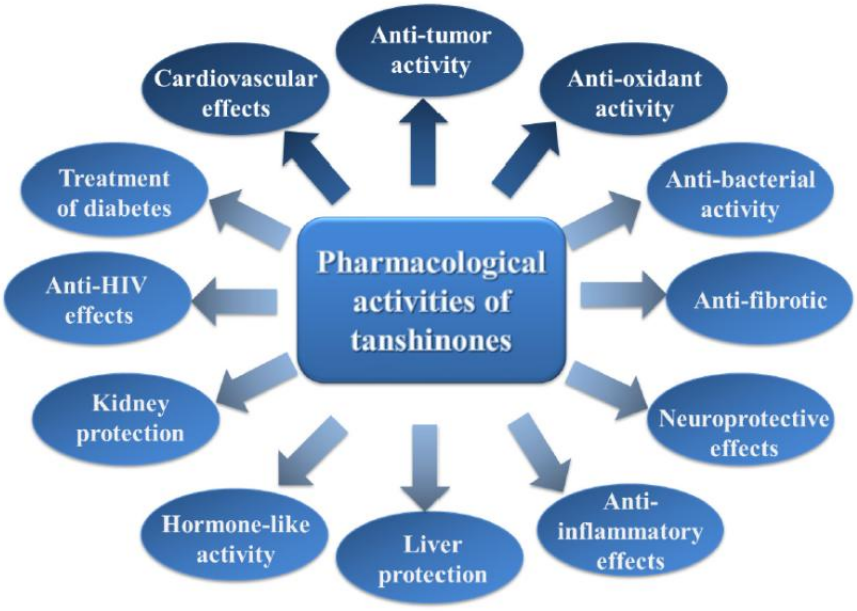


Figure 1. 10 Pharmacological activities of tanshinones.

Table 1. 5 List of diterpenes produced by *in vitro* culture of different *Salvia* species (1)

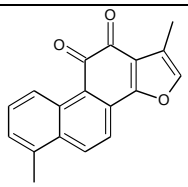
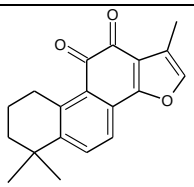
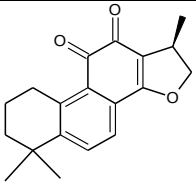
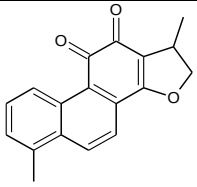
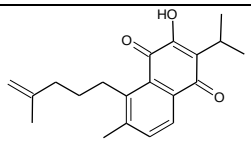
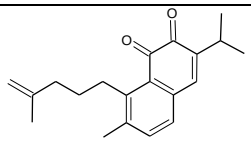
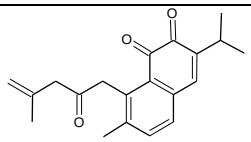
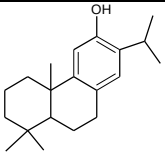
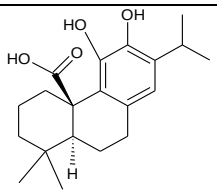
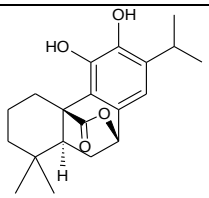
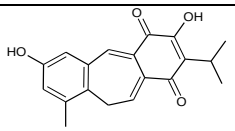
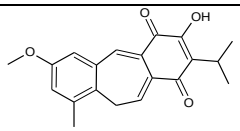
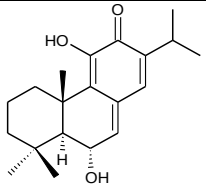
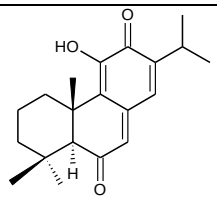
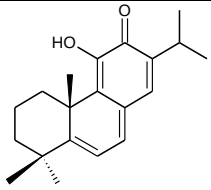
N.	Name	Chemical structure	Salvia species	*In vitro* culture	References
1	Tanshinone I		*S. miltiorrhiza*	HR CC	(Zhi & Alfermann, 1993) (H. Chen et al., 1997)
			S. castanea	HR	(B. Li et al., 2016)
2	Tanshinone IIA		*S. miltiorrhiza*	HR CC	(Zhi & Alfermann, 1993) (H. Chen et al., 1997)
			S. castanea	HR	(B. Li et al., 2016)
3	Cryptotanshinone		*S. miltiorrhiza*	HR CC	(Zhi & Alfermann, 1993) (H. Chen et al., 1997)
			S. castanea	HR	(B. Li et al., 2016)
4	Dihydrotanshinone I		*S. miltiorrhiza*	HR	(Zhi & Alfermann, 1993)
			S. castanea	HR	(B. Li et al., 2016)
5	Salvipisone		*S. sclarea*	HR	(M. Vaccaro et al., 2014)
6	Aethiopinone		*S. sclarea*	HR	
7	1-oxoaethiopinone		*S. sclarea*	HR	
8	Ferruginol		*S. sclarea*	HR	(M. Vaccaro et al., 2014)
			S. miltiorrhiza	HR	(Zhi & Alfermann, 1993)

Table 1.5 List of diterpenes produced by *in vitro* culture of different *Salvia* species (2)

8	Carnosic acid		*S. officinalis.* L	C CC	(Santos-Gomes et al., 2003)
				MP	(Izabela Grzegorczyk et al., 2005)
10	Carnosol		*S. officinalis.* L	C CC	(Santos-Gomes et al., 2003)
				MP	(Izabela Grzegorczyk et al., 2005)
11	Demethylfruticuline A		*S. corrugata*	RS MP Apex Leaves	(Bisio et al., 2016)
12	Fruticuline A		*S. corrugata*	RS Apex Leaves	(Bisio et al., 2016)
13	Taxodone		*S. austriaca*	HR	(Ł Kuźma et al., 2017)
14	Taxodione		*S. austriaca*	HR	(Ł Kuźma et al., 2011)
15	15-deoxy-fuerstione		*S. austriaca*	HR	(Ł Kuźma et al., 2017)

HR: hairy roots, CC: cell culture, C: callus, MP: micropropagated plants, RS: regenerated shoots.

1.5. Strategies to increase biosynthesis of diterpene on *Salvia species*

There are several parameters that would be taken in consideration to control the optimal *in vitro* production of bioactive secondary metabolite from plant. The sucrose level, exogenous growth hormone, the nature of the nitrogen source and their relative amounts, light, temperature and the presence of chemicals can all affect growth, total biomass yield, and secondary metabolite production (Nussbaumer et al., 1998; Rhodes et al., 1994), to increase metabolite production in the plant and to enhance its qualitative value for fresh produce, enriched food, or as a raw ingredient for feed/food and pharmaceutical products (Poulev et al., 2003; Smetanska, 2008). All these opened up a new area of research that could have important economic benefits for pharmaceutical industry.

For the *Salvia* species, the chemical composition of medium culture, the differentiation or not of tissues, the using of elicitor, the interaction with bacteria o fungi and the reduction of extracellular secondary metabolite through absorption represents some *in vitro* strategies to increase bioactive diterpenes production.

1.5.1. Effects of growth condition

The *in vitro* growth of plant organ, tissues and cells occurs in a suitable medium containing all the required elements. These ingredients of medium composition affect the biomass accumulation and secondary metabolite formation. External factors such as carbon source, nitrogen source, growth regulators, pH of the medium, temperature, light and oxygen are considered easy to regulate the expression of plant secondary metabolite pathways (Akula et al., 2011; S. R. Rao et al., 2002; Yue et al., 2016).

Effect of salt: The most used *in vitro* media are Murashige-Skoog (MS), which has a very high concentration of nitrate (NO^{-3}), potassium (K^{+}), and ammonia (NH^{+4}) as well as Gamborg (B5) (Gamborg et al., 1968) and 6,7-V medium (Veliky et al., 1970), with the level of inorganic nutrients in the medium lower than those in the MS medium. The salt's composition can affect the biomass growth and secondary metabolite production. In general, high ammonium ion concentrations inhibit secondary metabolite formation, while the lowering of ammonium

nitrogen increases it (Efferth, 2018). Zhi & Alfterman, in 1993, showed that diterpenes accumulation in the hairy roots of *S. miltiorrhiza* were higher in the absence of ammonium nitrate (19 mg diterpenes/g DW) than in its presence (7 mg diterpenes/g DW). In the *Ti transformed* cell suspension cultures, the B5 medium supported the best growth while the 6,7-V medium promoted tanshinone production (H. Chen et al., 1997). More recently, L. Liu et al. (2018), demonstrated that the hairy roots of *S. castanea* were more adaptive to Phosphate deficiency than those of *S. miltiorrhiza* in terms of biomass production, secondary metabolites and antioxidant activity. The elements such as Cu^{2+}, Zn^{2+}, Mg^{2+} in low concentration and Fe^{2+} and Mn^{2+} in high concentration would all advance the biosynthesis of tanshinone II A in the adventitious root culture of *S. miltiorrhiza* (X. Guo et al., 2005).

The composition of the medium or selection of medium also played a vital role in elicitation process (P. M. Naik et al., 2016; Patel et al., 2013).

Effect of hormones: Cytokinins types and concentration have different effects depending on the type of metabolite. On *S. miltiorrhiza* hairy root culture, the presence abscisic acid (ABA) and thidiazuron (TDZ) promote the increase of tanshinone I and cryptotanshinone 5-fold and 7.5-fold over the control respectively (Gupta et al., 2011). In addition, Santos-Gomes et al. (2003), showed that from *S. officinalis* L., carnosol content reached the highest value of 30.5±0.42 mg/g DW in suspension cultures supplemented with 1.5 mg/l zeatin and carnosic acid (12.7±2.96 mg/g DW) in calli culture supplemented with 1.5 mg/l 6-benzylaminopurine. However, there were some traces of carnosol and carnosic acid in the suspension cultures supplemented with 0.5 mg/l KIN (Kinetin).

Effect of sucrose: Initial sucrose concentration that represent the principal carbon source in plant culture, had a significant influence on the growth of transformed cell cultures of *S. miltiorrhiza*; growth was fastest in the medium containing 30 g/L sucrose, and the growth was obviously reduced at 60 g/L sucrose and totally inhibited at 100 g/L sucrose (H. Chen et al., 1997). The level of sucrose has been shown to affect the productivity of secondary metabolite accumulation in cultures (S. R. Rao & G. A. Ravishankar, 2002). In Ti-transformed *S. miltiorrhiza* cell suspension culture, the tanshinone production was better in the 30 g/L sucrose medium

than that in the 20 g/L or 40 g/L sucrose medium. However, much higher tanshinone production was observed in the 100 g/L sucrose medium although the growth of cells was almost completely inhibited under this condition (H. Chen et al., 1997).

Effect of Environmental Factors: In the natural world, light plays an important role in promoting plant growth and inducing or regulating plant metabolism (Abbasi et al., 2007; Ali et al., 2014; C. Liu et al., 2006; L. Yang et al., 2018). On the other hand, light causes an inhibitory effect on the accumulation of secondary metabolites such as nicotine and shikonin (Tabata et al., 1974). The green cell aggregates of *S. miltiorrhiza* transferred to the fresh B5 medium containing 4 g/L yeast extract and cultivated under darkness produced more tanshinones than their counterparts cultivated under continuous illumination, which suggested the inhibitory effect of light on tanshinone production in the cell cultures (H. Chen et al., 1997).

Effect of vitamins: Hitoshi Miyasaka et al. (1987), showed that the production of cryptotanshinone and ferruginol increased with an increase of thiamine concentration and 1-5 mg/l of thiamine HCl was the optimal concentration for the production of these compounds.

1.5.2. Elicitation

Plants and *in vitro* cultured plant cells show physiological and morphological responses to microbial, physical or chemical factors which are known as 'elicitors' (Chandra et al., 2011). The term elicitor was originally used for molecules capable of inducing the production of phytoalexins, but it is now defined as a substance for stress factors which, when applied in small quantity to a living system, it induces or improves the biosynthesis of specific compound which do have an important role in the adaptations of plants to a stressful conditions (Radman et al., 2003). It may include abiotic elicitors such as metal ions and inorganic compounds and biotic elicitors from fungi, bacteria, viruses or herbivores, plant cell wall components as well as chemicals that are released at the attack site by plants upon pathogen or herbivore attack (Soundarapandian et al., 2010). **Table 1.6** classifies the elicitors according to their nature.

Elicitation is a widely known method, which aims to enhance the production of secondary metabolites and has been described for many plant species. Discovery of elicitors has opened up a new approach to secondary metabolite production.

All culture types (cell, callus, root, hairy root, whole plant, shoots, and seedling) can be exposed to elicitor treatments. Methyl jasmonate, jasmonic acid, and salicylic acid are the most commonly used elicitors. Moreover, polysaccharides, such as pectin, dextran, chitin, chitosan, and alginate, are frequently utilized as elicitors for triggering secondary metabolites in plant cell and tissue cultures (Nartop, 2018).

Several parameters such as elicitor concentration and selectivity, duration of elicitor exposure, age of culture, cell line, growth regulator, nutrient composition, and quality of cell wall materials are also important factors influencing the successful production of secondary metabolite (Ganapathi et al., 1990). For example, a low concentration cannot be sufficient to stimulate secondary metabolite and a high concentration can decrease the growth of biomass or cause the death of plant. Elicitation at the right stage of culture, with elicitors applied alone or in combination are also the regulating factors responsible for enhancement of secondary metabolites (Chandra & Chandra, 2011).

Table 1. 6 Elicitors classification based on their nature (Naik & Al–Khayri, 2016).

Elicitors																
Biotic				Abiotic												
Polysaccharide	Yeast extract	Fungal	bacterial	Physical					Chemical			Hormone			Plant signal compound	
				UV radiation	Osmotic stress	Salinity	Drought	Thermal stress	Heavy metals	Mineral sals	Gaseous toxins	ABA	TDZ		Jasmonic acid	Salicylic acid

Generally speaking, elicitor induced stress results in the activation of several defense-related genes or inactivation of non-defense-related genes, transient phosphorylation/dephosphorylation of proteins, expression of enzymes whose information can be used to ascertain the biosynthetic pathways of many secondary metabolites. Nowadays, there are continued effort to understand molecular and genetic mechanisms involved to the process of elicitation.

1.5.2.1. Biotic elicitors

1.5.2.1.1. Microorganism - roots interaction

A wide range of microorganisms that need not be pathogens have been tried in cultures for elicitor induced production of secondary metabolites. Rhizosphere microbes are best known to act as biotic elicitors, which can induce the synthesis of secondary products in plants (Soundarapandian & Dhandayuthapani, 2010). The great parts of these microorganisms are endophytes (microorganisms that colonize the interior of host plants without causing apparent disease) and there were used in co-culture with their host plant tissues by some researchers as efficient stimuli leading to the production of secondary metabolite. In some case these microorganisms had more or less a direct interaction with the plant tissues and the quantity of inoculums is more important for induce elicitation action. However, not all the endophytes have the ability to co-culture with their host plant tissues for a long time, which depends on the toxicity of the fungal isolate. In most cases, those fungi were fabricated into elicitors with their toxicity removed, which can also stimulate the secondary metabolites of the host plants (Giauque et al., 2013). Entophyte has a symbiotic relationship with plants, which may experience a long evolutionary and interacting communication. **Table 1.7** report the effect of microorganism interaction and their extracts on diterpenes production in *in vitro Salvia* hairy roots cultures.

- **Root–bacteria interaction**

Actinomycetes are a numerous and widely distributed group of soil microbes, constituting to about 10 to 50% of the soil microflora community (Adegboye et al., 2012; Tyc et al., 2017), and other researchers have reported them to be important producers of secondary metabolites

(Olanrewaju et al., 2019). They have been widely studied for their ability to modulate relationships between plants and biotic/abiotic stresses, often producing valuable secondary metabolites that can affect host physiology (Shweta et al., 2013). A soil actinomycete strain, designated *Streptomyces pactum* Act12, has been isolated from the drought, low-temperature, and high-latitude environment on the Qinghai-Tibet Plateau (J. Zhao et al., 2011). Most *Streptomyces* are efficient rhizosphere and rhizoplane colonizers. They can also be endophytes colonizing inner tissues of host plants (de Jesus Sousa et al., 2016).

Y. Yan, Zhang, Yang, et al. (2014), showed that *S. miltiorrhiza* hairy roots under the treatment of 2% and 4 % *Streptomyces pactum* Act12 for 14 days, strongly promoted the synthesis of tanshinones, most significantly enhanced the concentration of CT 13.21 and **33.63 times** more than that of the control at a treatment of 2% and 4 % *Streptomyces* respectively as well as 8.42 and 15.31 for DT-I, 11.77 and 10.36 for T-IIA and 5.58 and 5.67 for T-I concentrations. ACT12's promotional effect on T-I was smaller than on several other tanshinone substances due to the fact that the concentrations of the four tanshinone substances were elevated significantly. The 2% and 4 % ACT12 treatments caused the concentrations of total tanshinone in the *S. miltiorrhiza* hairy roots to become as high as 9.19 and 12.61 times that of the control, respectively. This system is regarded as a highly efficient strategy, which may generate valuable secondary metabolites and provides a new type of elicitor for future research regarding plant secondary metabolic regulation.

Plant growth-promoting rhizobacteria (PGPR) are bacteria living in the rhizosphere, able to colonize the root system (rhizosphere, rhizoplane, or roots) of plants. These bacteria are beneficial to plant growth by facilitating the nutrient uptake and producing and supplying growth-promoting substances such as phytohormones, and plant protection (Barea et al., 2005; Paterson et al., 2016). *Bacillus cereus* is one of the most common rhizobacterial species that have been shown to enhance plant resistance to bacterial and fungal pathogens (Barea et al., 2005; Kloepper et al., 2004).

The research conducted by some authors (J.-Y. Wu et al., 2007; J.-L. Zhao et al., 2010), showed that living *B. cereus* cells inoculated into the *S. miltiorrhiza* hairy root culture on Day 0

stimulated dramatically the total tanshinone accumulation in roots but suppressed the hairy root growth. However, the growth of root biomass is promoted if the inoculation was performed in the late growth stage (J.-L. Zhao et al., 2010). The culture inoculated with 0.2% (OD value of 0.5) bacteria on day 0 attained the highest tanshinone content of **2.67 mg/g dw** and the highest volumetric yield of 10.4 mg/L (on day 28), which were 13.5- and 7.6-fold of those in the control, 0.20 mg/g dw and 1.40 mg/L, respectively (J.-Y. Wu et al., 2007). Few years later, the tanshinone accumulation was enhanced most dramatically by the bacterial concentration of 2.5% (OD value of 1.0) inoculated on Day 0 for only 7 days, to 2.78mg/g dw, which was about 18-fold higher than in the control 0.15mg/g dw. In addition, the highest volumetric tanshinone yield 22.4 mg/L, which was about 12-fold of the control yield 1.82 mg/L, was attained with the same bacterial concentration inoculated for 7 days into the culture on day 18 (J.-L. Zhao et al., 2010).

Y. Yan, Zhang, Zhang, et al. (2014), described that *Pseudomonas brassicacearum* sub sp. *Neoaurantiaca* (B1), isolated from both phloem and xylem of healthy *S. miltiorrhiza*, and then inoculated to *S. miltiorrhiza* hairy root culture at 0.025 % (OD value of 0.5) at the day 21 and maintained in co-culture for 9 days promoted the biomass growth. Additionally, promoted the increase of total tanshinone content in hairy roots, with the most obvious, reaching a 3.7-fold compared with the control, most obviously increasing DT-I and CT content (19.2-fold and 11.3-fold, respectively) compared to the control. In consequence, *S. miltiorrhiza* hairy roots have inhibition effects on growth of endophytic bacteria B1, this is mainly caused by the antibacterial effect of tanshinone substances in hairy roots.

- **Interaction endophytic fungal – hairy roots**

Zhai et al. (2018), investigated the effects of the live endophytic fungus *Chaetomium globosum* D38 on tanshinones biosynthesis in *S. miltiorrhiza* hairy roots and showed that, administration of D38 on day 18 significantly enhanced the contents of dihydrotanshinone I (DT-I) and cryptotanshinone (CT) by 8 fold and 14.9-fold respectively compared to the control.

1.5.2.1.2. Microorganism extract.

- **Fungal extract (*Extract of mycelium and polysaccharide)***

Trichoderma species control fungal pathogens by acting both as a microbial antagonist and by inducing localized and systemic plant defense responses (Harman et al., 2004). *Trichoderma atroviride* D16 endophytic fungus from the root of the medicinal herb *S. miltiorrhiza* Bunge is reported for the first time to produced tanshinone I and tanshinone IIA in rich mycological medium (Ming et al., 2012).

Ming et al. (2013), showed that both EM (extract of mycelium) and PSF (polysaccharide fraction) from *Trichoderma atroviride* D16 treatment were responsible for promoting hairy root growth caused a substantial alteration in the abundance of the four tanshinones. Among the four tanshinones species, DT-I and CT were most dramatically stimulated by EM and PSF on day 18. The content of DT-I in hairy roots treated with 300 mg/L EM was **~35-fold** that of the control (1.338 mg/g DW versus 0.039 mg/g DW), and the content of CT treated with 150 mg/l EM was almost **83-fold** over the control (3.061 mg/g DW versus 0.037 mg/g DW). Similarly, the content of DT-I and CT in hairy roots with treatment with 180 mg/L PSF were respectively **~23-fold** (1.216 mg/g DW versus 0.052 mg/g DW) and **~66-fold** (3.496 mg/g DW versus 0.053 mg/g DW) than that of the control.

Further, under the action of extract of mycelium (EM) from *C. globosum* D38, the content of dihydrotanshinone I and cryptotanshinone reached the highest level which were 21-fold and 19.8-fold compared to the control group, at a dose of 60 mg/L and 90 mg/L respectively (Zhai et al., 2018).

Chitosan and chitin are cell wall structural components found in many fungi. However, chitosan is a hydrophilic biopolymer obtained by N-deacetylation of chitin, and can be applied as an antimicrobial agent (Rabea et al., 2003). Zhao et al., (2010), showed that application of 100 mg/l chitosan in *S. miltiorrhiza* cell culture induce 3.4-fold (0.27 mg/g vs 80 µg/g of the control) the production of T-I.

- **Bacterial Extracts.**

Bacterial extracts consist of biological mixtures prepared from autoclaved and centrifuged microorganism cultures, without identification of the active compounds (Ramirez-Estrada et al., 2016). The bacterial extract of *Bacillus cereus* enhanced slightly both the hairy root growth and tanshinone biosynthesis when it was fed to the *S. miltiorrhiza* hairy root culture growing on MS medium on day 21. Wu et al., 2007 described that bacterial extract 100 µg/mL applied on day 0 and harvested 28 days after the application, enhanced root weight by nearly 50% and the TT content of roots increased by about twofold compared to the control. Furthermore, J.-L. Zhao et al. (2010), illustrated that the precedent concentration of bacterial extract 10 times more (1 g/L) applied on Day 0 and 7, and harvested 7 days after, decreased drastically the hairy root biomass by 5.21 g DW/L and 7.54 g DW/L to the control 12.2 g DW/L and increased significantly the tanshinone content by about 12-fold to 2.04 mg/g dw and 2.00 mg/g dw versus 0.17 mg/g dw in the control.

Additionally, **Coronatine** (Cor) an analog of methyl jasmonic acid is a polyketide effector molecule produced by *Pseudomonas syringae* pv tomato strain DC3000 (Pst DC3000), binds the same receptor and acts as a structural agonist of JA-Ile (J. Yan et al., 2013); firstly described as an elicitor by Weiler et al. (1994).

M. C. Vaccaro et al. (2017), showed that 0.1 µM of coronatine, applied for **28 days on** *S. sclarea* hairy root culture, allowed to extract 103.32 ± 2.10 mg/L of aethiopinone, corresponding approximatively to a **24-fold** increase over the basal content of control hairy roots (4.40 ± 0.13 mg/L). This content was higher than the 16-fold increase (73.29 ± 0.11 mg/L) induced by elicitation with MJ for the same elicitation time, and greater than the final yield obtained after 7 days with this elicitor **(24.08 ± 0.79 mg/L).** Interestingly, Cor enhanced the content of carnosic acid with the maximum yield obtained after elicitation for 28 days (36.75 ± 2.20 mg/L), corresponding to about an 18-fold increase above the basal content of control hairy roots (**Table 1.9**).

- **Yeast extract**

For decades, scientists are using yeast extract as one of the biotic elicitors. Despite limited knowledge of the composition and mechanism of action of yeast, autoclaved solutions providing cell wall fragments are widely used as elicitors to enhance plant secondary metabolite production, mainly in plant cell or hairy root cultures (Ramirez-Estrada et al., 2016).

Treatments of transformed and not transformed *S. miltiorrhiza* cell culture with yeast extract lead mainly to an inhibition of biomass accumulation (see **table 1.14**). Despite these, Ti transformed cell cultured in fresh 6,7-V medium containing 20 g/L sucrose with 4 g/L yeast extract for 8 days, stimulate the production of total tanshinone (CT and T-IIA) productivity of **22.2 mg/L** versus trace amounts into the control cultured in fresh B5 medium (H. Chen et al., 1997). When cultured in a MS-NH_4 medium (MS without ammonium nitrate, containing 30 g/L sucrose) yeast elicitor (4 g/L) for 18 days, this production increased from trace amounts in the control to **12.23 mg/L** (G.-J. Li et al., 2003). Treatments of 18 days old of not transformed *S. miltiorrhiza* cell culture with yeast extract 100 mg/L for 7 days decreased the biomass production by no more than 50% (5.1–5.5 g/L versus 8.9 g/L), but increased the total tanshinone content to **2.30 mg/g**, about 11.5-fold versus that of the control (0.20 mg/g) and more drastically CT content about **2,011 µg/g** which represent 34 fold of the control level (60 µg/g) (J-L. Zhao et al., 2010). This last result is in agreement with that of normal cell suspensions of *S. miltiorrhiza* in which cryptotanshinone production was stimulated only after cell growth was suppressed (H. Miyasaka et al., 1989). However, 4 days old of Ti transformed cells treated with 0.1% (v/v) YE, decreased the biomass accumulation than that in the control (13.1 vs 14.4 g dw/L at day 5) while the cryptotanshinone production enhanced greatly (**11.5** vs 0 mg/L) (H. Chen et al., 1999).

Table 1.8 illustrates the Effects of yeast extract on diterpenes production in *in vitro Salvia* hairy root cultures. Unlike cell cultures, the application of yeast extract on hairy roots non present only the inhibition of biomass production. The treatment of 18 days olds hairy roots of *S. miltiorrhiza* for 9 days with YE 100 mg/L increased the volumetric TT (CT, T-I, T-IIA) 7.62 mg/L about 4.3-fold over the control (M Shi et al., 2007), and a treatment for 4 days increased TT about ~2.2 mg/g DW which represent 3.1-fold over the control (X Ge et al., 2005). However, the

treatment with YE 25 mg/L increased this amounts to 9.92 mg/L about 5-fold over the control (Jian-Yong Wu et al., 2008). The TT (CT, T-I, T-IIA) significantly increased when 18 days olds hairy root was treated with 100 mg/L for 12 days reached 13.7 mg/L about 3.8-fold over the control (Q Yan et al., 2005). D. Yang et al. (2018), investigated the tanshinones production when *S. miltiorrhiza* hairy root was applied to 200 mg/L of YE and showed that the cryptotanshinone was the diterpene mostly. Chen et al., (2001), reported that the intracellular content of cryptotanshinone increased from 0.001% to as much as 0.096% of dry weight. The amount of CT was enhanced most dramatically, increasing from 0.124 to 0.914 mg/g dry weight.

The treatment of *S. castanea* Diels *f. tomentosa* Stib with optimum concentration 200 mg/L yeast extract benefited both the growth status and tanshinone accumulation. The cryptotanshinone content was improved to 2.84 ± 0.33 mg/g DW at most in the hairy root cultures. Moreover, an evident activation of T-IIA showed a sustainable promotion to 2.52±0.67 mg/g DW and DT-I to 1.95±0.09 mg/g DW (B. Li et al., 2016). The T-I content was slightly responsive to elicitation (B. Li et al., 2016); however, it was drastically enhanced about 37.14-fold of the control levels (D. Yang et al., 2018).

1.5.2.2. Abiotic elicitors

Abiotic elicitation involves triggering the synthesis of phytochemical compounds in plants using chemical or physical stimuli (Owolabi et al., 2018).

1.5.2.2.1. Chemical elicitors

- **Heavy metals**

Various heavy metal elements have been used to induce specialized metabolites in *S. miltiorrhiza*, such as Lanthanum, cereus, silver, cobalt and cadmium (L. H. Bian et al., 2016; Han et al., 2015; C. Zhang et al., 2004; J-L. Zhao et al., 2010) as reported in **Table 1.10** Among them, Ag^{+} is the most common elicitor at this fact. However, it causes a doses-dependent depression of *S. miltiorrhiza* hairy roots and cell growth (C. Zhang et al., 2004; J-L. Zhao et al., 2010).

S. miltiorrhiza cell suspension exposed to Ag^{+} 25 µM for 7 days significantly increased total tanshinone (CT, T-I, and T-IIA) content to 2.04 mg/g, about 10-fold versus that of the control (0.20 mg/g) with large amount of CT 1,817.5 µg/g about 30-fold of the control 59.9 µg/g (J-L.

Zhao et al., 2010). Recently, Yu et al., (2019), showed that cryptotanshinone was 18.07 times higher than that of the control on day 7 after treatment with Ag^{+} 60 µM (**Table 1.14**).

- **Plant signal compounds**

- **Methyl jasmonate**

Jasmonates are defined as "hormones" because they elicit cellular responses at low concentrations distant from their site of synthesis (Baenas et al., 2014; Rohwer et al., 2008). Methyl jasmonate acts as an efficient elicitor of secondary metabolite production across the plant kingdom, particularly those involved in a developmental process and defense responses (De Geyter et al., 2012). It was used by some researchers to increase the *in vitro* production of diterpenes in some *Salvia* species (**Table 1.9**).

Some study of elicitation of *S. miltiorrhiza* hairy root by MJ showed that the ideal concentration to avoid a good production of different tanshinone less than 4-fold to the control in is around 100 to 150 µM (Hao et al., 2015; Liang et al., 2012; Xing et al., 2018). However, these results are too different to the previous, which reported that after elicitation with MJ, cryptotanshinone and tanshinone IIA were 23.8 fold and 6.2 fold higher than that of the control respectively (X. Wang et al., 2007). In cell culture, MJ showed only a moderate or insignificant stimulating effect on tanshinone accumulation in normal and transformed *S. miltiorrhiza* cell cultures (H. Chen & Chen, 1999; J-L. Zhao et al., 2010).

The total diterpenoid content of *S. sclarea* hairy root in shake flask elicited with 125 µM of MJ for 7 days, was twice the diterpenoid content of control roots. Additionally, the diterpenoid content of *S. sclarea* at the same treatment using bioreactor as growth system, reached (67.5 mg/g DW) about 6-fold higher than that of the non-elicited and 2.4 times higher in relation to MJ-treatment roots maintained in shake flasks (28.16 ± 1.2 mg/g DW). Among all these diterpene, aethiopinone was found to be the main diterpenoid synthesized by roots of *S. sclarea* treated by MJ. Its content was 40 mg/g DW, **9.1-fold** over the control and about **60%** of the total diterpenoids (Ł. Kuźma et al., 2009). M. C. Vaccaro et al. (2017), found that 100 µM MJ applied for 7 days on hairy root of *S. sclarea*, appeared to be more effective in triggering aethiopinone accumulation (9.72 ± 0.08 mg/g DW) corresponding to a **25-fold** increase over the

content of untreated hairy roots (0.38 ± 0.07 mg/g DW). However, using the sprinkle bioreactor system as growth culture had a greater effect on hairy roots biomass and secondary metabolite production can be of practical significance in using the culture. This may probably be due to a higher root biomass and consequently, exposing more receptors to the elicitor (D'Amelia et al., 2017).

MJ showed only a moderate stimulating effect on tanshinone accumulation in *S. castanea* Diels *f. tomentosa* Stib. hairy root culture (B. Li et al., 2016).

- **Salicylic acid**

Numerous studies on the *Salvia* genus have applied SA elicitation to enhance diterpene production (**Table 1.11**). SA has been reported as an effective elicitor for hairy root and cell culture of *S. miltiorrhiza.* Hao et al. (2015), showed total tanshinone production after application of SA on *S. miltiorrhiza SmGGPPS* overexpression hairy roots was only 1.63-fold of the mimic treatment control. J-L. Zhao et al. (2010) and Yu et al. (2019), treated *S. miltiorrhiza* non transformed cell culture for 7 days with 100 and 200 µM, the content of cryptotanshinone produce were 6.5-fold and 4.39-fold higher than the control respectively (**Table 1.14)**.
Kračun-Kolarević et al. (2015), reported a significant increase carnosol and carnosic acid (CA) in shoots culture of *S. officinalis.* Carnosol and carnosic acid contents were higher (3.8 and 1.4 times respectively) in 4 weeks-old control than in 1 week old explants. SA treatment increased carnosol production from 2 mg/g DW (in 1 week old control explants) to 14 mg/g DW (in 4 weeks old shoots growing on 150 µM).

- **Others elicitors on *S. miltiorrhiza* hairy roots**

Table 1.11 reports the effect of others chemical compounds on diterpenes production in *in vitro Salvia* hairy roots cultures. The application of 100 µM sodium nitroprusside (SNP), a donor of NO, resulted in a significant increase production contents of tanshinone I, cryptotanshinone, dihydrotanshinone I and tanshinone IIA in hairy roots by 80, 170, 60 and 180% above the control level, respectively (Yang et al., 2012). The application of *β*-aminobutyric acid as elicitor at the dose of 2 mM, caused a significant increase of total tanshinone content of 1.09 mg/g DW, about 4.5 times that of the control, 0.24 mg/g DW (X. Ge et al., 2005). The addition of PEG

2% increased tanshinone I, cryptotanshinone, dihydrotanshinone I, and tanshinone IIA in the hairy roots to 0.9, 2.1, 3.9, and 2.0 mg/L compared to the control 0.7, 1.3, 3.2, and 1.5 mg/L, respectively (Yang et al., 2012). Smoke-water (SW) 1:1000 (v/v) has been found very effective for enhancement of Tanshinone I accumulation in hairy roots of *S. miltiorrhiza* after 3 days of elicitation (J. Zhou et al., 2018).

1.5.2.2.2. Physical elicitors (effect of light irradiation)

The spectral quality, intensity, and period of light irradiation may affect plant cell and tissues cultures. Several studies demonstrated the involvement of light irradiation in secondary metabolite accumulation (**Table 1.12**). *S*. *miltiorrhiza* hairy root exposed to UV–B irradiation, show increase in the production of three tanshinones (CT, T-IA and T-IIA) reached the peak (0.38 mg/g DW), 1.8-fold than that of control. Among the three tanshinones, cryptotanshinone was most and reached maximum (0.13 mg/g DW), 3.4-fold than that of control after 40 min of UV-B irradiation (C. H. Wang et al., 2016). Recently, different 1- and 3-weeks LED light spectra treatments could regulate tanshinones in *S. miltiorrhiza* hairy roots. Blue light decreased T-IIA content via down regulation of keys enzymes involve in the biosynthetic process (I. J. Chen et al., 2018).

1.5.2.3. Combination or synergic effect of elicitors

Some studies have shown that the production of secondary metabolites can be enhanced or potentiated by incorporation of multiple elicitor treatment over the culture period more than a single treatment in the hairy root cultures of *S. miltiorrhiza* (Cheng et al., 2013; Hao et al., 2015; Kai et al., 2010; C. H. Wang et al., 2016). In hairy roots and cell culture of *S*. *miltiorrhiza,* large part of research investigated the combination of yeast extract with other elicitors (**Table 1.13** and **1.15**).

The *Ti-transfomed S. Miltiorrhiza* cell cultures treated with combination of yeast extract and salicylic acid for 15 days, enhanced the total tanshinone production (CT and T-IIA) to reached the highest levels with **15.07 mg/L**, while there was 12.23 mg/L in yeast extract treatment and hardly detectable amount of tanshinone in the control and salicylic acid treatments (G.-J. Li et al., 2003). Few years earlier, H. Chen and Chen (1999), had showed that in cell suspension when

SA 200 µM was supplemented one day before the yeast elicitor it enhanced the cryptotanshinone formation from 13.8 mg/L to **18.9 mg/L** (an increase of 37%). Combinations of two elicitors (YE + Ag^{+} or YE + Cd^{2+}) and three elicitors (YE + Ag^{+} + Cd^{2+}) in the *S. miltiorrhiza* cell culture increased tanshinone content about 20% and 40% respectively in combination compared with that of a single elicitor (J-L. Zhao et al., 2010).

Sorbitol widely used as osmoticum in plant cell and tissue cultures, had a slight enhancing effect on root growth (Zaker et al., 2015) and can have a positive effect on secondary metabolite production (Hong et al., 2012). The combination of yeast extract with sorbitol (100 mg/l + 50 g/L respectively) added on day 21 and maintained for 9 days in suspension hairy roots culture of *S*. *miltiorrhiza,* decreased the biomass production and increase more significantly tanshinone (CT, T-I, T-IIA) production **16.3** versus 1.77 mg/L compared with the control (M Shi et al., 2007). A year later, Jian-Yong Wu and Shi (2008), investigated that combination of yeast extract 25 mg/l with sorbitol + 50 g/L increased biomass production and increased more drastically tanshinone (CT, T-I, T-IIA) production by sevenfold (from 0.2 to 1.6 mg/g dry weight) and the volumetric yield by 13-fold (from 1.95 to **27.4 mg/L**) compared to the batch control culture.

The combination of methyl jasmonate with a pretreatment for 40 min with UV-B on *S. miltiorrhiza* hairy roots, increased considerably total tanshinone (CT, T-I, T-IIA) to **28.21 mg/L**, a 4.9-fold over the control (C. H. Wang et al., 2016). Other good tanshinone production, was obtained by combination of yeast extract pretreated for 3 days with of β-aminobutyric acid, reached the amount **20.1 mg/l** (X. Ge & J. Wu, 2005); or for 2 days with Ag+ with CT being enhanced dramatically (X Ge & J Wu, 2005).

1.5.2.4. Elicitation and nutrient or medium feeding or renewal

Precursor feeding has been an obvious and popular approach to increase secondary metabolite production in plant cell cultures. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor (S. R. Rao & G. Ravishankar, 2002).

In principle, elicitation is to induce or stimulate the secondary metabolite of plant cells but it is not favorable for cell growth (primary metabolism). However, suppressed biomass growth has been observed in hairy root cultures treated by abiotic and biotic elicitors, especially at

relatively larger doses. This unfavorable effect could be reduced or completely eliminated during the process of elicitation by nutrient or medium feeding or renewal, thus improving the secondary metabolite production more effectively (Diwan et al., 2011; C. Wang et al., 2001; Q Yan et al., 2005; J.-L. Zhao et al., 2014). The Ag^+ treatment 15 µM caused a depression of *S. miltiorrhiza* hairy root 9.6 g DW/L versus 12.1 g DW/L of the control. The replenishment of sucrose and fresh medium before the addition of Ag^+ to the culture medium, sustained the hairy root growth by 18.9 g DW/L and 22.3 g DW/L versus 12.1 g DW/L of the control respectively; and total tanshinone production by 36.6 mg/L and 55.7 mg/L versus 24.4 mg/L and 7.3 mg/L to Ag^+ treatment and control respectively (C. Zhang et al., 2004).

It was found that, sorbitol was mainly a strong stimulator of the tanshinone production and had beneficial effects on the *S. miltiorrhiza* hairy root growth (M Shi et al., 2007; Jian-Yong Wu & Shi, 2008). Yeast extract was also found as an effective stimulator of the tanshinone production and presented a positive or negative effect on hairy roots growth according to growth conditions, its concentration and duration of elicitation (H. Chen et al., 2001; X Ge & J Wu, 2005; M Shi et al., 2007; Jian-Yong Wu & Shi, 2008; Q Yan et al., 2005; D. Yang et al., 2018). Both a frequent elicitor (Sorbitol+YE) challenge and a sufficient nutrient supply to the *S. miltiorrhiza* hairy roots (from 10 mL every 5 days) were essential for maintaining the secondary metabolite production through the extended fed-batch process without affecting biomass. Most notable was the ultrahigh tanshinone content attained in the OS+YE treated culture, 18.1 mg/g DW (equivalent to 1.8 wt % or **143.9 mg/L of** TT yield), about 11.5-fold of that in the process with a single treatment (1.57 mg/g dw), and nearly 100-fold of that without any elicitor treatment (0.2 mg/g dw). Moreover, the root fragments released from the roots in the OS+YE treated culture had an even higher tanshinone content, 110 mg/g dw or 11 wt.% (Jian-Yong Wu & Shi, 2008).

1.5.2.5. Production with in situ adsorption

In nature, some secondary metabolites are hydrophobic and stored intracellularly either in the cytosol or cell vacuole. They are minimally secreted in the culture medium and may appear in the culture broth as a result of cell lysis (Chandra & Chandra, 2011; Chiang et al., 2007). However, some secondary metabolites produced in the cell can be released into the culture

medium. In immobilized cultured cells of *S. miltiorrhiza*, much of the cryptotanshinone was released into the medium, while most of the ferruginol was retained in the cells (Hitoshi Miyasaka et al., 1986). The production of target metabolites can be limited by both feedback inhibition of accumulated synthesized metabolites and their degraded compounds (Q. Yan et al., 2011). The introduction of an in situ product removal mechanism, such as a solid adsorbent to the culture medium can often effectively induce product release from plant cells and increase productivity (Vuong et al., 2014).

To increase metabolite production in plant cell culture, various adsorbents have been used for the solid/liquid two-phase system. The addition (2 g of X-5 resin in a nylon bag in each 200-mL flask) recovered a major portion of tanshinones (CT, T-I, T-IIA) from the roots, 70–94 %. Moreover, the combination of the macroporous polystyrene resin(X-5 adsorption) with double YE elicitation (added to the culture on days 30 and 40 at 100 mg/L) led to a significant increase in the tanshinone yield. To prolong biomass growth and secondary metabolite production, medium renewal, YE elicitation, and resin replacement were performed every 10 days for three times (days 30, 40, and 50). Fascinating, the root biomass was increased to 30 g/L DW (vs. 8–10 g/L DW in batch mode) and the volumetric tanshinone yield to 87 mg/L (about a 15-fold increase), with 76 % adsorbed to the resin. The volumetric productivity of total tanshinone reached 1.46 mg/L per day, more than 7 times that of the batch culture (Q Yan et al., 2005).

Table 1. 7 Effect of microorganism interaction and their extracts on diterpenes production in *in vitro Salvia* hairy roots cultures.

Salvia	*A. Rhizogenes*	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	ATCC 15834	MS medium	0.3 g fresh root in 25 mL medium at 110–120 rpm in dark and at 25°C	Bacterium *B. Cereus*	0.2%	0	28	Inhibit	TT	0.20 mg/g DW	2.67 mg/g DW	**1,250**	(J.-Y. Wu et al., 2007)
									Volumetric TT	1.40 mg/L	10.4 mg/L	660	
				Bacterium *B. Cereus* (extract)	100 mg/L	0	28	Increase 50%	TT	0.17 mg/g DW	0.54 mg/g DW	100	
									Volumetric TT	1,15 mg/L	5.35 mg/L	250	
				Bacterium *B. Cereus* (root–bacteria co-culture supernatant collected on day 21)	5 ml (25% v/v)	0	28	Inhibit	TT	0.16 mg/g DW	0.79 mg/g DW	400	
									Volumetric TT	0.85mg /L	3.95 mg/L	360	
	ATCC 15834	MS medium	0.3 g fresh hairy roots 25 mL medium at 110–120 rpm in the dark and at 25 ◦C	*B. Cereus* (Bacterial cells)	2.5%]	0	7	Inhibit 50%	TT	0.15 mg/g DW	2.78 mg/g DW	**1,700**	(J.-L. Zhao et al., 2010)
						18	7	Increase	Volumetric TT	1.82 mg/L	22.4mg/L	**1,100**	
				B. Cereus (extract)	1g/L	0	7	Inhibit 50%	TT	0.17mg/g DW	2.04 mg/g DW	**1,100**	
				Bacterial polysaccharide (BPS)	100 mg/L	18	7	Increase 20%	TT	0.19 mg/g DW	1.59 mg/g DW	700	
									Volumetric TT	2,11mg/L	21.6 mg/L	900	
	C58C1	1/2 B5 medium	1.0 g fresh roots in 100mL medium at 180 rpm in dark and at 25°C	*Trichoderma atroviride* D16 extract of mycelium (EM)	300 mg/l	21	18	Increase	DT-I	0.039 mg/g DW	1.338 mg/g DW	**3,400**	(Ming et al., 2012)
					150 mg/L	21	18	Increase	CT	0.037 mg/g DW	3.061 mg/g DW	**8,200**	
				Polysaccharide fraction (PSF)	180 mg/L	21	18	Increase	DT-I CT	0.052 mg/g DW 0.053 mg/g DW	1.216 mg/g DW 1.216 mg/g DW	**2,200** **6,500**	
	ATCC 15834	MS medium	Hairy roots (0.3 g FW) into 40 mL medium at 120 rpm at 25°C and in the dark	Endophytic bacteria *Pseudomonas brassicacearum* sub sp. n*eoaurantiaca* (B1)	0.025 %	21	9	Increase	TT DT-I CT	Not published Not published Not published	Not published Not published Not published	270 **1,820** **1,030**	(Y. Yan, Zhang, Zhang, et al., 2014)
	ATCC 15834	MS medium	Hairy roots (0.3 g FW) into 40 mL medium at 120 rpm at 25°C and in the dark	*Streptomyces pactum* Act12	2%	21	14	No difference	T-I T-IIA DT-I CT TT	Not published Not published Not published Not published Not published	Not published Not published Not published Not published Not published	458 **1,077** 742 **1,221** 819	(Y. Yan, Zhang, Yang, et al., 2014)
					4%			Slightly inhibit 9.89%	T-I T-IIA DT-I CT TT	Not published Not published Not published Not published Not published	Not published Not published Not published Not published Not published	467 936 **1,431** **3,263** **1,161**	
	C58C1	1/2 B5 medium		*Chaetomium globosum* D38	Not published	Not published	18	Not published	CT DT-I	Not published Not published	Not published Not published	**1,390** 700	(Zhai et al., 2018)
				Chaetomium globosum D38 Extract of Mycelium (EM)	90 mg/L	-	18	Not published	CT	Not published	Not published	**1,880**	
					60 mg/L	-	18		DT-I	Not published	Not published	2,000	

Table 1. 8 Effect of yeast extract on diterpenes production in *in vitro Salvia* hairy root cultures.

Salvia	*A. Rhizogenes*	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	ATCC 15834	6, 7-V medium	0.3 g fresh root segments in 50 mL medium at 140 rpm in darkness and at 25°C.	YE	Not published	20 after renewal the medium	7	Increase	Intracellular CT	0.01 mg/g DW	0.96 mg/g DW	**9,500**	(H. Chen et al., 2001)
		MS medium	0.3 g fresh root in 25 mL medium at 110–120 rpm in dark and at 25°C	YE	100 µg/mL	18	4	Inhibit	TT	~0.7mg/g DW	~2.2 mg/g DW	210	(X Ge & J Wu, 2005)
		MS medium	0.3 g fresh weight of roots into 40 mL medium at 110–120 rpm	YE	100 mg/L	18	12	Increase about 30%	Volumetric TT	3.59 mg/L	13.7 mg/L	280	(Q Yan et al., 2005)
				YE					CT	0.124 mg/g DW	0.914 mg/g DW	640	
									TT	0.46 mg/g DW	1.37 mg/g DW	200	
						18, one day after X-5 resin was applied	repeated medium renewal, elicitation and resin replacement: thrice (days 30, 40, 50)	Increase	Volumetric TT (CT, T-I, T-IIA)	5.74 mg/L	87.5 mg/L	**1,420**	
									TT (CT, T-I, T-IIA)	0.56 mg/g DW	0.668 mg/g DW	**19**	
		MS medium	0.3 g fresh weight of roots into 40 mL medium at 110–120 rpm	YE	100 mg/L	21	9	Inhibit	TT	146.4 µg/g dw	732.4 µg/g dw	400	(M Shi et al., 2007)
									Volumetric TT	1,77 mg/L	7.62 mg/L	330	
		MS medium	0.2 g fresh weight of roots in 25 mL medium at 110–120 rpm	YE	25 mg/L	21	9	Inhibit	TT (CT, T-I, T-IIA)	0.2 mg/g DW	0.96 mg/g DW	380	(Jian-Yong Wu & Shi, 2008)
									Volumetric TT	1.96 mg/L	9.92 mg/L	400	
		6,7-V medium	0.2 g fresh hairy roots into 50 mL at 110 rpm in dark and at 25°C	YE	200 mg/L	18	6	Increase	DT-I CT T-I	Not published Not published Not published	Not published Not published Not published	238 378 100	(D. Yang et al., 2018)
S. castanea Diels	ATCC 15834	6,7-V medium	Hairy roots were cultured in into 50 mL medium	YE	200 mg/l	18	7	Increase 73%	CT T-IIA DT-I	Not published Not published Not detected	2.84 mg/g DW 2.52 mg/g DW 1.95 mg/g DW	737 177 -	(B. Li et al., 2016)
		6,7-V medium	0.2 g fresh hairy roots into 50 mL at 110 rpm in dark and at 25°C	YE	200 mg/L	18	6	Increase	DT-I CT T-I T-IIA	Not published Not published Not published Not published	Not published Not published Not published Not published	129 800 **3,614** 232	(D. Yang et al., 2018)

Table 1. 9 Effect of methyl jasmonate and coronatine on diterpenes production in *in vitro Salvia* hairy roots cultures

Salvia	*A. Rhizogenes*	Culture mediu m	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	ATCC 15834	6,7-V mediu m	Not published	MJ	Not published	18	9	Not published	CT, T-IIA	Not published Not published	0.571 mg/g DW 1.563 mg/g DW	**2,280** 520	(X. Wang et al., 2007)
	ATCC 15834	MS mediu m	Hairy roots (0.3 g) in 50 mL medium at 110–120 rpm on an orbital in darkness at 25ºC	MJ	100 µM	18	6	Inhibit	T-I CT T-IIA DT-I	Not published	Not published	200 290 100 30	(Liang et al., 2012)
		1/2 MS mediu m	Hairy roots into 50 mL medium at 100 rpm in the dark and at 25°C	MJ	100 µM	60	1.5	Not published	TT (T-I, T-IIA, CT, DT-I)	3.55 mg/g DW	11.33 mg/g DW	210	(Hao et al., 2015)
	R1601	MS mediu m	Hairy roots in 50 ml medium at 120 rpm and at 25°C	MJ	100 µM	18	7		TT CT T-IIA	Not published Not published Not published	1.46 mg/g DW 0.65 mg/g DW 0.36 mg/g DW	220 520 380	(C. H. Wang et al., 2016)
	ATCC 15834	6,7-V mediu m	Fresh hairy roots 0.2 g into 50 mL liquid medium at 110 rpm in the dark and at 25 °C.	MJ	100 µM	18	9	No difference	CT DT-I	Not published Not published	Not published 0.35 mg/g DW	76 81	(Xing et al., 2018)
S. sclarea	LBA 9402	1/2 B5 mediu m	Fresh hairy root 8.3 g into sprinkle bioreactor containing 2L at 26 ◦C under continuous cool-white fluorescent light (40 µmolm^{-2} s^{-1})	MJ	125µM	23	7	Increase	Ferruginol Salvipisone Aethiopinone 1-Oxoaethiopinone	0.9 mg/g DW 3.3 mg/g DW 4.4 mg/g DW 2.9 mg/g DW	9.7 mg/g DW 12.6 mg/g DW 40.0 mg/g DW 5.2 mg/g DW	980 280 810 80	(Ł. Kuźma et al., 2009)
	ATCC 15834	1/2 MS mediu m,	Hairy roots (1 g) into 100 mL at 120 rpm, at 23°C and in the dark	MJ	100 µM	21	7	No difference	Aethiopinone	0.38 mg/g DW	9.72 mg/g DW	**2,400**	(M. C. Vaccaro et al., 2017)
									Ferruginol	1.93 mg/L	56.13 mg/L	**2,800**	
							28	Inhibit	Aethiopinone	4.40 mg/L	73.29 mg/L	**1,500**	
									Ferruginol	Not published	55.48 mg/L	-	
				Corona-tine	0.1 µM		7	No difference	Aethiopinone	0.38 mg/g DW	2.57 mg/g DW	600	
							28	No difference	Aethiopinone	4.40 mg/L	103.32 mg/L	**2,300**	
									Ferruginol	Not published	103.01 mg/L	**5,300**	
									Carnosic acid	Not published	36.75 mg/L	**1,700**	
S. castanea Diels	ATCC 15834	6,7-V mediu m	Hairy roots were cultured in into 50 mL medium	MJ	200 µM	18	7	Increase	T-IIA	Not published	1.8 mg/g DW	99	(B. Li et al., 2016)
									CT	0.34 mg/g DW	0.51 mg/g DW	50	

Table 1. 10 Effect of Ag^{+} on diterpenes production in *in vitro Salvia* hairy roots cultures.

Salvia	A. Rhizogenes	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	ATCC 15834	MS medium	0.3 g fresh root in 25 mL medium at 110–120 rpm in dark and at 25°C	Ag^{+}	15 µM	18	12	Inhibit	TT	0.5 mg/g DW	2.3 mg/g DW	360	(C. Zhang et al., 2004)
									Volumetric TT	7.3 mg/L	25.3 mg/L	250	
									Volumetric CT	1.76 mg/L	12.6 mg/L	500	
				Sucrose + Ag^{+}	15 µM	Suc. on day 16 and Ag+ on day 20	9	Increase	Volumetric TT	Not published	Not published	54 compare to Ag^{+}	
				Medium renewal + Ag^{+}	15 µM	Suc. on day 16 and Ag+ on day 20	9	Increase	Volumetric CT Volumetric T-IIA	Not published Not published	Not published Not published	50 compare to Ag^{+} 120 compare to Ag^{+}	
		MS medium	0.3 g fresh root in 25 mL medium at 110–120 rpm in dark and at 25°C	Ag^{+}	30 µM	18	4	Inhibit	TT	Not published	Not published	20	(X Ge & J Wu, 2005)
		6,7-V medium	0.2 g fresh hairy roots in 50 mL medium at 110 rpm in the dark and at 25 °C	Ag^{+}	15 µM	18	6	No difference	DT-I T-I CT T-IIA TT	Not published Not published Not published Not published Not published	0.60 mg/g DW 0.82 mg/g DW 0.34 mg/g DW 0.42 mg/g DW 2.37 mg/g DW	156 46 42 340 120	(Xing et al., 2015)
		6,7-V medium	0.2 g fresh hairy roots into 50 mL at 110 rpm in dark and at 25°C	Ag^{+}	15 µM	18	6	No difference	DT-I CT T-I	Not published Not published Not published	Not published Not published Not published	65 64 85	(D. Yang et al., 2018)
	ACCC 10060	6, 7-V medium	0.3 g fresh root in 25 mL medium at 110 rpm in dark and at 25°C	La^{3+}	0.01 mM	18	1	Not published	T-I T-II CT	Not published Not published Not published	Not published Not published Not published	74.9 40.9 92.4	(L. Bian et al., 2016)
	ATCC 15834	6, 7-V medium	Hairy roots (0.2 g fresh mass) in 50 mL medium at 110 rpm in darkness and at 25±1ºC	Ce^{3+}	50 µM	18	3	No difference	DT-I CT	Not published Not published	0.875 mg/g DW 0.271 mg/g DW	271 393	(Han et al., 2015)
S. castanea Diels	ATCC 15834	6,7-V medium	Hairy roots were cultured in into 50 mL medium	Ag^{+}	15 µM	18	7	Increase	T-IIA	Not published	Not published	80	(B. Li et al., 2016)
		6,7-V medium	0.2 g fresh hairy roots into 50 mL at 110 rpm in dark and at 25°C	Ag^{+}	15 µM	18	6	Increase	CT T-I	Not published Not published	Not published Not published	233 67	(D. Yang et al., 2018)

Table 1. 11 Effect of others chemical compounds on diterpenes production in *in vitro Salvia* hairy roots cultures (1)

Salvia	*A. Rhizogenes*	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza		1/2 MS medium	Hairy roots into 50 mL medium at 100 rpm in the dark and at 25°C	SA	100 µM	60	1.5	-	TT (T-I, T-IIA, CT, DT-I)	3.55 mg/g DW	5.95 mg/g DW	63	(Hao et al., 2015)
	ATCC 15834	MS medium	0.2 g of fresh roots in 25 mL medium at 110–120 rpm in the dark and at 25ºC	Sorbitol	70 g/L	21	6	Slightly increase	TT	165.1 µg/g DW	723.6 µg/g DW	350	(M Shi et al., 2007)
					50 g/L				Volumetric TT	1.69 mg/L	8.18 mg/L	380	
	ATCC 15834	MS medium	0.2 g fresh weight of roots in 25 mL medium at 110–120 rpm	Sorbitol	50 mg/L	21	9	Increase	TT (CT, T-I, T-IIA)	0.2 mg/g DW	0.67 mg/g DW	235	(Jian-Yong Wu & Shi, 2008)
									Volumetric TT	1.96 mg/L	10.5 mg/L	436	
						21 with repeated Sorbitol and nutrient feeding every 5 days	From days 21 to day 60	No difference	TT (CT, T-I, T-IIA)	0.36 mg/g DW	3.22 mg/g DW	794	
									Volumetric TT	3.01 mg/L	39.4 mg/L	**1,200**	
	ATCC 15834	MS medium without NH_4NO_3	0.3 g fresh hairy roots into 50 mL at 110–120 rpm in darkness and at 25°C	Abscisic acid (ABA)	200 µM	18	6	Inhibit	T-I CT DT-I T-IIA	0.7 mg/g DW 1.3 mg/g DW 3.2 mg/g DW 1.5 mg/g DW	1.1 mg/g DW 1.6 mg/g DW 3.9 mg/g DW 2.2 mg/g DW	110 170 60 90	(Yang et al., 2012)
	ATCC 15834	Not published	Not published	ABA	210 µM	20	7	Not published	T-I CT DT-I T-II A	Not published Not published Not published Not published	Not published Not published Not published Not published	440 850 50 80	(Yang et al., 2012)
	BCRC 15010	B5 liquid medium	(1.5 cm, ~5.4 mg dw) root in 70 ml medium at 100 rpm in the dark and at 25±2°C	(ABA) abscisic acid	1.0 mg/L	0	84	No difference	T-I T-IIA CT TT	0.216 mg/g DW 0.120 mg/g DW 0.374 mg/g DW 0.71 mg/g DW	0.452 mg/g DW 0.341 mg/g DW 1.038 mg/g DW 1.831 mg/g DW	100 200 200 150	(Gupta et al., 2011)
				TDZ	1.0 mg/L			Increase	T-I T-IIA CT TT	0.216 mg/g DW 0.120 mg/g DW 0.374 mg/g DW 0.71 mg/g DW	0.393 mg/g DW 0.195 mg/g DW 1.310 mg/g DW 1,957 mg/g DW	100 50 250 170	

Table 1.11 Effect of other elicitors on diterpenes production in *in vitro Salvia* hairy root cultures (2)

Salvia	*A. Rhizogenes*	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	ATCC 15834	MS medium	0.3 g fresh root in 25 mL medium at 110–120 rpm in dark and at 25°C	BABA	2 mM	18	4	Inhibit	TT (CT, T-I, T-IIA)	0. 24 mg/g DW	1.09 mg/g DW	350	(X. Ge & J. Wu, 2005)
		MS medium	Hairy roots (0.3 g) in 50 mL medium at 110–120 rpm on an orbital in darkness at 25ºC	SNP	100 µM	18	6	No difference	T-I, CT, T-IIA DT-I	Not published	Not published	80 170 180 60	(Yang et al., 2012)
		MS medium without NH_4NO_3	0.3 g fresh hairy roots into 50 mL at 110–120 rpm in darkness and at 25°C	Polyethylene glycol (PEG),	2 % (w/v)	18	6	Inhibit	T-I CT DT-I T-IIA	0.7 mg/g DW 1.3 mg/g DW 3.2 mg/g DW 1.5 mg/g DW	0.9 mg/g DW 2.1 mg/g DW 3.9 mg/g DW 2.0 mg/g DW	70 100 60 70	(Yang et al., 2012)
	ACCC 10060	6, 7-V medium	at 110–120 rpm at 25 ± 1 °C in darkness	Smoke–water (SW)	SW 1:1000 (v/v)	18	3	Not published	T-I	Not published	Not published	230	(J. Zhou et al., 2018)

Table 1. 12 Effect of physic elicitors on diterpenes production in *in vitro Salvia* hairy root cultures

Salvia	*A. Rhizogenes*	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	R1601	MS medium	Hairy roots in 50 ml medium at 120 rpm and at 25°C	UV-B	40 µW/cm2	18	40 min		TT CT	Not published Not published	0.38 mg/g DW 0.13 mg/g	80 240	(C. H. Wang et al., 2016)
	LBA1334	1/2 B5 medium	0.3 g fresh hairy roots in dish under LED light intensity of 110 µmol m-2 s-1 for 16 h per day	Light 9R (100% Red) 9IR (100% far-red) 6R3IR (R:IR=100:26)	Light intensity of 110 µmol m-2 s-1 for 16 h per day.	28	7	No difference	T-IIA	Not published Not published Not published	Not published Not published Not published	 40 20 60	(I. J. Chen et al., 2018)

Table 1. 13 Effect of combination of elicitors on diterpenes production in *in vitro Salvia* hairy roots cultures

Salvia	*A. Rhizogenes*	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	ATCC 15834	MS medium	0.3 g fresh root in 25 mL medium at 110–120 rpm in dark and at 25°C	BABA + YE	1 mM + 100 µg/mL	BABA on day 15 and YE on days 18	3 days after adding YE	Inhibit	TT (CT, T-I, T-IIA)	1 mg/g DW	2.26 mg/g DW	126	(X. Ge & J. Wu, 2005)
									Volumetric TT	3.2 mg/L	20.1 mg/L	530	
	ATCC 15834	MS medium	0.2 g of fresh roots in 25 mL medium at 110–120 rpm in the dark and at 25ºC	YE + Sorbitol	100 mg/L + 50 g/l	21	9	Inhibit	TT (CT, T-I, T-IIA)	146.4 µg/g DW	1481.6 µg/g DW	900	(M Shi et al., 2007)
									Volumetric TT	1.77 mg/L	16.3 mg/L	800	
	ATCC 15834	MS medium	0.2 g fresh weight (fw) of roots in 25 mL medium at 110–120 rpm	YE + Sorbitol	25 mg/L + 50 g/L	21	9	Increase	TT (CT, T-I, T-IIA)	0.20 mg/g DW	1.57 mg/g DW	700	(Jian-Yong Wu & Shi, 2008)
									Volumetric TT	1.96 mg/L	27.8 mg/L	**1,300**	
						21 with repeated Sorbitol +YE and nutrient feeding every 5 days	From days 21 to day 60	No difference	TT (CT, T-I, T-IIA)	0.36 mg/g DW	18.1 mg/g DW	**4,900**	
									Volumetric TT	3.01 mg/L	**143.6 mg/L**	**4,670**	
	ACCC 10060	6,7-V medium	The rootwas transferred to 50 mL medium at 80 rpm in dark and at at 25°C	YE + Ag+	2.5 mg/mL + 100 µM	18	5	Not published	DT-I	Not published	Not published	760	(Cheng et al., 2013)
									CT	Not published	Not published	700	
				YE + MJ	2.5 mg/mL + 200 µM	18	5	Not published	TT	Not published	Not published		
				MJ + Ag+	200 µM + 100 µM	18	5	Not published	TT,	Not published	Not published	170	
									DT-I	Not published	Not published	380	
									CT	Not published	Not published	290	
				YE+ Ag+ + MJ	2.5 mg/mL + 100 µM + 200 µM,	18	5	Not published	DT-I	Not published	Not published	800	
									CT	Not published	Not published	660	
	R1601	MS medium	Hairy roots in 50 ml medium at 120 rpm and at 25°C	UV-B + MJ	40 µW/cm2 after 100 µM MJ	18	UV (40 min) MJ (9 days)	Inhibit	CT	Not published	0.97 mg/g DW	890	(C. H. Wang et al., 2016)
									T-I	Not published	0.93 mg/g DW	510	
									TT	Not published	2.26 mg/g DW	570	
									Volumetric TT	Not published	**28.21 mg/L**	4.9-fold	
							UV (40 min) MJ (7 days)		T-IIA	Not published	0.46 mg/g DW	380	

Table 1. 14 Effect of elicitors on diterpenes production in *in vitro Salvia* cell cultures

Salvia	Transformed or non	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated	Yield of control	Yield after elicitation	% Increase over the control	References
S. miltiorrhiza	*A. tumefaciens*	MS medium without NH_4NO_3	Fresh cell culture (ca. 0.4 g) into 20 mL medium in darkness at 25 °C	YE	4 g/L	7	15	Inhibit	TT (CT, T-IIA)	trace	12.23 mg/L	-	(G.-J. Li et al., 2003)
		B5 medium	3 g fresh weight in 100 mL at 140 rpm in darkness at 25°C for 16 days	YE	4 g/L	0	8	Inhibit	TT	Trace	20.1 mg/L	-	(H. Chen et al., 1997)
		6,7-V medium		YE	4 g/L	0	8	Inhibit	TT	Trace	22.2 mg/L	-	
		B5 medium	3.5 g cell into 50 mL medium at 140 rpm at dark and at 25°C	YE	0,1% (v/v)	4	5	Inhibit	CT	0 mg/L	11.5 mg/L	-	(H. Chen & Chen, 1999)
	Non	MS with 0.5 mg/L 2,4-D, 1 mg/L KIN	Into 100 mL of at 27 ± 0.5 °C at 120 rpm in the dark.	SA	200 µM	18	7		CT	Data not shown	Not published	339	(Yu et al., 2019)
				Ag^+	60 µM	18	7		CT	Data not shown	Not published	**1,707**	
		MS medium with 2 mg/L 2,4-D and 2 mg/L 6-BA	0.3 g fresh cells in 25 ml medium at 110–120 rpm, at 25°C in the dark	SA	100 µM	18	7	Inhibit	CT	59.9 µg/g DW	390.0 µg/g DW	550	(J-L. Zhao et al., 2010)
									T-IIA	57.6 µg/g DW	72.8 µg/g DW	30	
				Co	50 µM	18	7	Slightly inhibit	CT	59.9 µg/g DW	263.7 µg/g DW	340	
				Cd	25 µM	18	7	Inhibit	CT	59.9 µg/g DW	1,854.0 µg/g DW	**3,000**	
									T-IIA	57.6 µg/g DW	369.0 µg/g DW	540	
							6		Volumetric TT	1.9 mg/L	10.7mg/L	460	
				SO	50 g/L	18	7	Inhibit	CT	59.9 µg/g DW	584.6 µg/g DW	880	
									T-IIA	57.6 µg/g DW	83.0 µg/g DW	40	
				Chitosan	100 mg/L	18	7	Inhibit	CT	59.9 µg/g DW	597.2 µg/g DW	900	
									T-I	81.6 µg/g DW	276.0 µg/g DW	240	
				MJ	100 µM	18	7	Inhibit	CT	59.9 µg/g DW	299.8 µg/g DW	400	
									T-IIA	57.6 µg/g DW	82.6 µg/g DW	40	
				Ag^+	25 µM	18	7	Inhibit	CT	59.9 µg/g DW	1,817.5 µg/g DW	**2,900**	
									T-IIA	57.6 µg/g DW	225.8 µg/g DW	290	
							6		Volumetric TT	1.9 mg/L	11.7 mg/L	520	
				YE	100 mg/L	18	7	Inhibit	CT	59.9 µg/g DW	2,011.4 µg/g DW	**3,300**	
									T-IIA	57.6 µg/g DW	190.3 µg/g DW	230	
							6		Volumetric TT	1.9 mg/L	9.2 mg/L	380	

Table 1. 15 Effect of combination of elicitors on diterpenes production in *in vitro Salvia* cell cultures

Salvia	Transformed o non	Culture medium	Growth condition	Elicitors	Conc. of elicitor	Elicitated on day	Time of elicitation (days)	Biomass growth	Diterpenes stimulated			% Increase over the control	References
S. miltiorrhiza	*A. tumefaciens*	B5 medium	3 g fresh weight in 100 mL at 140 rpm in darkness at 25°C for 16 days	YE + light (L)	4 g/L + 4000 lux.	0	8	Data not shown	TT	No detectable	1.92 mg/L	-	(H. Chen et al., 1997)
				YE + dark (D)	4 g/L	0	8		TT	No detectable	8.76 mg/L	-	
			3.5 g of fresh cell into 50 mL medium containing 20 g sucrose/L at 140 rpm in darkness and at 25°C.	YE + SA	YE 0,1% (v/v) + SA 200 µM	3 and 24 h after add YE	5	Inhibit	CT	0 mg/L	18.9 mg/L	37	(H. Chen & Chen, 1999)
	Non	MS medium without NH_4NO_3	Fresh cell culture (ca. 0.4 g) into 20 mL medium in darkness at 25 °C	YE + SA	0.12 mL + 200 µM	7	15	Inhibit	TT (CT, T-IIA)	Hardly detectable	15.07 mg/L	-	(G.-J. Li et al., 2003)

1.6. Plant material

1.6.1. *Salvia corrugata*

1.6.1.1. Classification

Kingdom: *Plantae*- Plants

Subkingdom: *Tracheobionta*- Vascular plants

Division: *Magnoliophyta*- Flowering plants

Class: *Magnoliopsida*- Dicotyledons

Subclass: *Asteridea*

Order: *Lamiales*

Family: *Lamiaceae*

Subfamily: *Nepetoideae*

Genus: *Salvia*

Subgenus: *Calosphace*

Specie: *corrugata* Vahl

Synonym: - *Alguelahuen gaudichaudii* Briq; *sphacele gaudichaudii* Briq.

1.6.1.2. Botanical description

Salvia corrugata is a perennial shrub native to America (Columbia, Peru, and Ecuador) (**Fig.1.11**), growing at 8000–9800 ft elevation. It was brought into horticulture about 2000 as a result of a collecting trip to South America in 1988. All the plants in cultivation today are from six seeds that germinated from that trip. *Salvia corrugata* reaches 9 ft in its native habitat, and 5–6 ft in cultivation. It has egg-shaped deeply corrugated evergreen leaves that are 4.5 in by 1.5 in, dark green on the top surface, and light veining with pale tan-colored fine hairs underneath. The brilliant purple-blue flowers are 1 in long, with a small dark purple and green calyx. The flowers grow in congested whorls, with 6–12 flowers on each 3–4 in inflorescence (Clebsch, 2003). *Salvia corrugata* is easily grown in Mediterranean coastal areas as ornamental purposes (Epling, 1939). This plant is used in traditional medicine by Ecuadorian indigenous ethnicities (Jara et al., 2013).

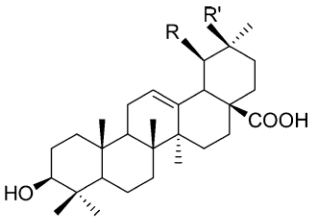
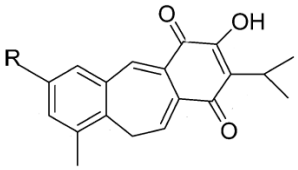
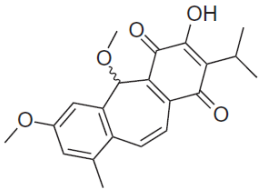
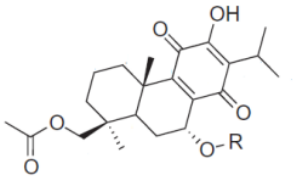
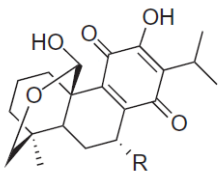
Figure 1. 11 Geographic distribution of *S. corrugata* (from: https://www.gbif.org/species/3905260).

1.6.1.3. Phytochemical composition of *Salvia corrugata*

The evaluation of phytochemical composition of *S. corrugata* was firstly carried out by (Bisio et al., 2008). The isolation and purification of leaf surface constituents (exudates) allowed the isolation of some terpenes compounds (**Table 1.16**) such as ursolic acid (**1**) in mixture with oleanolic acid (**2**) and oxidized diterpene quinones fruticuline A (**3**) and demethylfruticuline A (**4**) (Bisio et al., 2008). Few years later following compounds were isolated (**5**), (**6**), (**7**) by (Giacomelli et al., 2013).

A large number of these icetexane diterpenoids compounds are isolated from various species in the Salvia genus. Demethylfruticuline A, fruticuline A are the mains icetexane diterpenes constituents in the aerial part of *S. corrugata* Vahl (Bisio et al., 2008).

Table 1. 16 Chemical structure of terpenes isolated from exudates of leaves of *S. corrugata.*

Structures	-	-	Name
	**1)**	R= Me, R’ = H	Ursolic acid
	2)	R= H, R’ = Me	Oleanolic acid
	**3)**	R = Ome	Fruticuline A
	4)	R = OH	Demethylfruticuline A
	**5)**		Fruticuline C
	**6)**	R = CH	7a-methoxy-19-acetoxy-royleanone (7a-methoxy-12-hydroxy-19-acetoxy-11,14-dioxoabieta-8,12-diene)
	7)	R = Ac	7a,19-diacetoxy-royleanone (7a,19-diacetoxy-12-hydroxy-11,14-dioxoabieta-8,12-diene)
	**8)**	R = H	7-dehydroxy-conacytone

1.6.1.4. Biological activity of *Salvia corrugata*

The activity of the *S. corrugata* fruticuline A and demethylfruticuline A was assessed against 46 bacterial pathogens, mostly resistant to several primary antibiotics. The MIC for all the inhibited Gram-positive pathogens tested showed a very narrow distribution and ranged from 32 to 64 mg/L, regardless of their resistance patterns to other antibiotics. Demethylfruticuline A was shown to be highly bactericidal (>3 log10 CFU decrease within 24 h) against *Staphylococcus aureus* and S. epidermidis and bacteriostatic against *E. faecalis* and *E. faecium*. Fruticuline A manifested bacteriostatic activity against all tested strains (Bisio et al., 2008; Schito et al., 2011). Demethylfruticuline A and fruticuline A were more effective against *S. aureus* strains (>70% effect at subinhibitory concentrations) than against *S. epidermidis* in inhibiting slime synthesis. Against *E. faecalis*, demethylfruticuline A at subinhibitory concentration induced an inhibition of biofilm production of ca. 60%; fruticuline A was less active and more strain-dependent (Schito et al., 2011). Demethylfruticuline A induces anoikis, a type apoptosis induced in mammalian cells through a loss of cell adhesion mediated by CD36 (Monticone et al., 2010) and also caused apoptosis by inducing reactive oxygen species in mitochondria (Monticone et al., 2010).

1.6.1.5. Biotechnology applied to *Salvia corrugata*

According to literature, it exist only one study *in vitro* biotechnology from *S. corrugata*. As it was earlier described on establishment of *in vitro* culture of *S. corrugata*, analysis of the regenerated shoot showed the presence of both icetexanes with the yield of fruticuline A higher in the methanolic extract than in those of fresh leaves and fresh shoot tips. However, micropropagated plants contained only fruticuline A, while the callus contained trace amounts of both diterpenes (Bisio et al., 2016).

1.6.2. *Salvia tingitana*

1.6.2.1. Classification

Kingdom: *Plantae*- Plants

Subkingdom: *Tracheobionta*- Vascular plants

Division: *Magnoliophyta*- Flowering plants

Class: *Magnoliopsida*- Dicotyledons

Subclass: *Asteridea*

Order: *Lamiales*

Family: *Lamiaceae*

Subfamily: *Nepetoideae*

Genus: *Salvia*

Species: *tingitana* Etl.

Synonym: *Sclarea tingitana* (Etl.)

1.6.2.2. Botanical description

The name *S. tingitana* was formed and validated by Andreas Ernest Etlinger in 1777, although the plant to which it refers had been known for at least eighty years before. In those days, its origin was unknown or uncertain and, until very recently, there is evidence that it came originally from the Arabian region, although the epithet implies an African origin Tingi (ancient city of the Roman Province of Mauritania), today Tangier in Morocco. There is now evidence that it originally came from the Arab region (Foley et al., 2008).

S. tingitana is a perennial herbaceous species with a woody base up to 90-100 cm tall and as wide as many, with an intense aroma, sprinkled in all its parts with sparse and long hairs and sessile or pedicellate glands. Sturdy and erect quadrangular stems, with 2-4 cm long internodes. Leaves with a 4-6 cm petiole, absent in the leaves near the inflorescence: lamina ovata long fine at 15 cm and up to 10 cm broad, of gray-green color, with sub-corded base, wavy edge and irregularly lobed, with a strongly wrinkled upper surface and ribs marked on the lower side. The terminal inflorescences are about 30 cm long, generally with some basal branches, spaced between them of 2-3 cm generally formed by 6 pedicellated flowers: ovate-acuminate floral bracts 1-1.5 cm long. Triangular calyx 15-20 mm long, strongly hairy-glandy, green, spinulose in the fruit. Corolla 25-30 mm long, with a white or light lavender cut upper lip, white-yellowish concave lower lip (Cervelli, 2004).

CHAPTER 2 OBJECTIVES

The main objective of the present study is the improvement of the knowledge of two new *Salvia* species for the development of secondary metabolites described recently able to control human bacterial growth: *S. corrugata* and *S. tingitana*.

A very innovative system to produce secondary metabolites is the utilization of technologies that can be controlled for the growth parameters in order to have sterile biomass with well known growth speed. This is very important for scaling up the biomass production up to industrial level.

In this pathway, the first step is the evaluation and set up of the *in vitro* multiplication protocols together with the development of differentiated plant tissues (i.e. hairy root) or undifferentiated-ones (callus); the second step is the analysis of the growth kinetics that is the base for the set up of an elicitation protocol; then it is necessary to understand the scale up possibilities.

The study explore all the *in vitro* protocols to develop cell and hairy root biomass of these species and the scale up techniques in order to have high amount of material for the phytochemical analysis.

The experimental activities were planned and developed through a collaborative project between the Department of Pharmacy of University of Genoa and CREA-OF Sanremo.

CHAPTER 3 MATERIALS AND METHODS

3.1. Materials and methods *Salvia corrugata* Vahl

3.1.1. *Salvia corrugata* shoot *in vitro* development and elicitation

3.1.1.1. *In vitro* shoot culture establishment

Explants of *S. corrugata* plants grown in field and open air at the Agricultural Research Council Unit CREA-OF located in Sanremo (Italy) at latitude 43°49'05''N and longitude 07°45'30''E. were taken and sterilized as follows: washing with tap water for 15 min following by treatment with 1% of active chlorine supplemented with few drop of Tween 20 for 15 min. The explants were rinsed three times with sterile distilled water for 10' each. The sterile shoots were grown onto solid MS (**Annex 1**) medium, composed of macro and microelements, vitamins, 30 mg/L of sucrose and 8 g/L plant agar. The pH was adjusted using KOH and HCl to 5.7; about 62.5 mL medium was poured into 250 mL glass vessels before autoclaving at 1 atm and 121 °C for 20'. The shoots were grown at 23 ± 2 °C under day-night illumination regime (photoperiod 16:8) by white fluorescent Philips Master TL-D 36 W/840 lamp flux density 35 $\mu E.m^{-2}.s^{-1}$ and the medium was renewed monthly. In order to obtain shoot multiplication 0.3 mg/L of Benzyl adenine (BA) was added to the base medium (Mascarello et al., 2006).

3.1.1.2. Shoot elicitation in TIS (RITA®) bioreactor with methyl jasmonate

The experiment was carried out with *S. corrugata* shoot culture coming from solid MS medium supplemented with BA 0.3 $mg.L^{-1}$ (Mascarello et al., 2006). Fifteen explants with 3 internodes each, with an initial total fresh weight of about 0.85 g were cultured in 0.5 liter Temporary Immersion Systems (TISs) containing 150 mL of liquid MS medium supplemented with BA 0.3 mg/L. The sequence of immersion of shoots by liquid medium inside the bioreactor lasts 3 min every 3 hours. All the cultures were maintained in the growth chamber at 23 ± 2˚C under 16 hours light photoperiod. Light was provided by white fluorescent Philips Master TL-D 36W/840 lamp (photon flux density 35 $\mu E.m^{-2}.s^{-1}$). After 18 days three concentrations (50, 100 and 200 µM) of MJ (stock dissolved in ethanol 95%), were added after filtration (with Whatman filter 0.2 µm) to the 18-days-old shoot culture for 3 days. After that, the shoots were transferred in MS medium without hormone for one week before harvest on 28^{th} days. The control culture on day 21 was transferred without treatment in fresh MS medium without hormone for one week

before to be harvested. For each concentration, three bioreactors were prepared. The medium pH and the conductivity were measured after treatment with MJ on day 21 with the respective electrodes on a BASIC 20+ pH meter and a CM 35 conductivity meter. At the end of the culture on days 28, the fresh material was stored at -80˚C prior lyophilization. Shoot growth was determined on fresh and dry weight of control and elicited shoots. *In vitro S. corrugata* shoots obtained were extracted with methanol and the residue was evaporated under reduced pressure. The material was conserved at 4°C prior the chemical analysis to quantify fruticuline A and demethylfruticuline A as reported in Section **3.1.3.2.**

3.1.1.3. Shoot elicitation in glass vessel with light

In order to evaluate the effect of light as elicitor on the production of secondary metabolites, 4 weeks old shoots of *S. corrugata*, cultured on MS added with BA 0.3 mg/L were incubated at five different light intensities: 0; 30; 45; 80 and 100 $\mu E.m^{-2}s^{-1}$ supplied by fluorescent Philips Master TL-D 36W/840 lamp measured by luxometer (*LI-COR, inc.* QUANRUM/RADIOMETER/PHOTOMETER*, MODEL* LI-185B). The experimental design consisted in 4 replications with 28 explants each per each light treatment. After 3 weeks, the cultures were analyzed; data were recorder about fresh and dry weight. All the material was weighted and stored at -80°C prior lyophilization. The material was then analyzed as reported in Section **3.1.3.2.**

3.1.2. Establishment and analysis of hairy roots from *Salvia corrugata* Vahl.

3.1.2.1. A. rhizogenes strain culture

The hairy root transformation was performed with two strains of *Agrobacterium rhizogenes* (**Table 3.1**): *A. rhizogenes* wild type ATCC 15835 (glycerol stock from CREA-OF Sanremo) and the hypervirulent *A. rhizogenes* strain LBA 9402 (a gift from Prof. Laura Pistelli, University of Pisa, Italy). The bacteria were grown onto semi-solid (1.5% agar) YMB (Hooykaas et al., 1977) (**Table 3.2** A) and NB medium (**Table. 3.2** B) in dark. For each strain, a single bacterial colony was selected and transferred into 50 ml sterile polypropylene conical tube containing 10 mL of the same medium without agar for overnight growth at 28 °C and under agitation 120 rpm. Optical density was measured by spectrophotometer ($O.D._{600nm}$ = 0.3 for ATCC 15834 and 0.35 for LBA 9402).

Table 3. 1 Characteristics of A. rhizogenes strain used for transformation of S. corrugata.

***A. rhizogenes* strain**	**ATCC 15835**	**LBA 9402**
Growth medium	NB	YMB
Plasmid carried	pRi 15834	pRi LBA9402 (Satdive et al., 2006)
Opine group	Agropine	Agropine

Table 3. 2 Medium for bacterial growth (A) YMB and (B) NB

Composition of YMB	
Mannitol	10 g
K_2HPO_4	0.5 g
Yeast extract	0.4 g
$MgSO_4$. $7H_2O$	0.2 g
NaCl	0.1 g
pH	7.0 ± 0.2

(A)

Composition of Nutrient Broth (NB)	
Gelatin peptone	5g
Beef extract	3g
pH	6.8 ± 0.2

(B)

The indicated quantities are referred to a liter of liquid medium to obtain solid medium 15 grams per liter of agar must be added.

3.1.2.2. Co-cultivation and induction

The leaves of the second and third node of *S. corrugata*, grown in MS hormone-free medium for 4 weeks were isolated and wounded gently on both sides of the leaf tissue and on the central rib, immersed into 5 mL of the previous bacterial suspension and swirled for 20' at 24°C. The control of the experiment was carried out by treating explants with sterile distilled water for the same time and temperature. All sub-cultured explants were laid horizontally on sterile Petri plates with solid (0.8% agar) hormone-free MS medium and incubated at 24˚C in dark for 3 days. The explants were then transferred every 15 days for 3 passages onto MS medium containing cefotaxime (100 mg/L of cefotaxime was added for the strain ATCC 15834 while 500 mg/L was used for strain LBA 9402). The transformation efficiency after 30 days from the co-culture was determined by the number of explants that developed putative hairy root divided by the total number of explants used for transformation following this formula:

$$\%\ \text{of root induction} = \frac{\text{Number of explants with putative hairy root}}{\text{Total number of explants}} \times 100$$

3.1.2.3. Hairy roots clone selection and maintenance

Single roots with length more than 5 mm were excised from the leaf explants and transferred to single Petri dish with agarized MS medium containing the amount of cefotaxime for each bacterial strain, 100 mg/L for ATCC 15934 and 500 mg/L for LBA 9402. Among the different roots developed, only those with specific phenotypic characteristics of hairy roots (i.e. rapid branching, missing geotropism, fast growth rate and development of white hairs after the growth on hormone-free medium) were considered as putative HR material selected and maintained for further studies. After 30 days, the selected clones of root derived from ATCC 15834 and LBA 9402, based on length and number of branching were transferred on fresh MS medium containing cefotaxime concentration reduced respectively to 50 mg/L and 300 mg/L for other 30 days, after that cefotaxime was eliminated.

Root clones were sub-cultured onto the same solid medium every month. After two months of sub culturing without antibiotic, the axenic hairy roots were transferred to MS0 liquid medium and tested for liquid adaptability. For liquid culture, clones were cultured into 10 mL of a sterilized MS0 liquid medium in a 250 mL glass vessel and incubated at dark condition under agitation of 60 rpm.

3.1.2.4. Detection of Ri T-DNA integration

3.1.2.4.1. DNA extraction

Genomic DNA of the three selected clones of hairy roots SCO-HR-FA8, SCO-HR-FA13, SCO-HR-FL7 and of a negative control from non transformed roots (normal root from micropropagated plant) was extracted from 100 mg of plant tissue by Dneasy Plant Mini Kit (Qiagen®) (**Annex 2**). The DNA concentration was quantified using a NanoDrop® 2000 Spectrophotometer (Thermo scientific) (D'Angiolillo et al., 2012; Desjardins et al., 2010). Positive control was represented by plasmid DNA of *Agrobacterium rhizogenes* strain ATCC 15835 carried out according to the Klimyuk method (Klimyuk et al., 1993) (**Annex 3**). DNA samples were used as a template for PCR in order to determine the presence of the *rol*C gene in the T-DNA (i.e. root transformation) and *virC1* gene (i.e. presence/absence of bacterial contamination).

3.1.2.4.2. PCR analysis and electrophoresis of transgenic roots

The Polymerase Chain Reaction (PCR) was achieved in BIO RAD T100^{TM} Thermal cycler and was used to detect the Ri T-DNA integration in the plant genome. The analysis was performed to target two specific *A. rhizogenes* genes*: rolC* and *virC1* genes. The eventual amplification of *virC1* gene fragment indicates the presence of *A*. *rhizogenes* bacterial cells contamination or the residual presence of bacterium in the plant tissues; this was detected by amplification of a 326 bp using *virC1*-specific primers (PVIRC2775 5'-CTCGCTCAGCAGCAGTTCAATG-3' and PVIRC3101 5'-GACGGCAAACGATTGGCTCTC-3') (Vaira et al., 1995). The presence of the *rolC* gene was checked by PCR amplification of a 514 bp fragment (forward Primer (5'-CGACCTGTGTTCTCTCTTTTTCAAGC-3') and reverse primer (5'-GCACTCGCCATGCCTCACCAACTCACC-3')). Each reaction for the amplification of 514 bp fragment of the *rolC* gene, and 326 bp fragment of the *virC1* genes was performed with plant

DNA diluted 1:2 with distilled water and specific primer. The PCR reaction was performed in a final volume of 25 µL with 1 µL of DNA solution, 3 µL of PCR Rxn buffer (-$MgCl_2$), 0.9 µL of $MgCl_2$, 0.6 µL of dNTPs, 0.5 µL of each primer, 0.3 µL of platinium® Taq (DNA polymerase) and 18.2 µL of water (**Table 3.3**). The *rolC* PCR condition gene followed cycling program (Scorza et al., 1994) modified: initial denaturation at 94˚C for 1', 35 cycles at 94˚C for 30'' denaturation, 65˚C for 30'' annealing, 72˚C for 1' elongation and final elongation step of 5' at 72˚C (**Fig 3.1**). The absence of bacterial contamination of plant tissue was confirmed after PCR amplification of 326 bp fragment of the *virC1* gene using the same PCR program.

Table 3. 3 Amplification mixture composition

Solutions	Concentration	Volume (per unit)
Distilled water	----------	18.2 µL
PCR Rxn buffer (-$MgCl_2$)	----------	3 µL
$MgCl_2$	50 mM	0.9 µL
dNTPs	0.2 mM	0.6 µL
Primer 1	10 µM	0.5 µL
Primer 2	10 µM	0.5 µL
platinium® Taq (DNA polymerase)	5 U/µL	0.3 µL
MIXTURE (total)	----------	**25 µL**

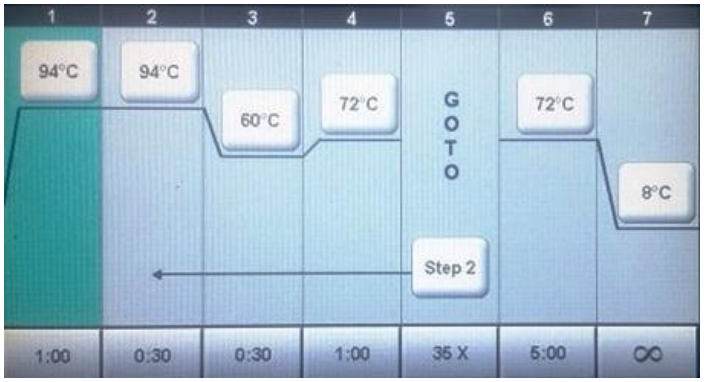


Figure 3. 1 Technical program for gene amplification**. Legend: 1: initial denaturation, 2: denaturation, 3: annealing, 4: elongation, 5: go to step 2 (35X), 6: elongation and 7: standby.**

Amplification products of these genes were detected after electrophoresis analysis on agarose gel 1.5% (**Annex 4**) in TAE buffer, and stained with ethidium bromide. The gel was introduced in the electrophoresis cell and immersed with TAE buffer solution. The comb was removed, leaving wells where 10 µL of each DNA samples (DNA amplified mixed with 2 µL of loading dye buffer) and 4µL of marker (GeneRuler^{TM} 1kb Plus 0.1 µg/µl) were loaded. DNA is run for 45 Min and at a voltage of 75 V. The amplification bands were detected by ultraviolet light.

3.1.2.5. Histochemical analysis of *Salvia corrugata* hairy root

3.1.2.5.1. Terpene staining

In order to detect the presence of terpene inside the diverse structure of plant tissues the Nadi protocol was used (Ibanez et al., 2010; López et al., 2018; Pistelli et al., 2020). Nadi specifically stains lipids in blue and terpenes in purple. The dye was prepared using α-naphthol (0.5 mL 1% v/v in 40% ethanol) and dimethylphenylenediamine-HCl (0.5 mL 1% v/v) in sodium phosphate buffer pH 7 (5 mL 50 mM) (DAVID Rand CARDE, 1964). The mechanism is a reaction resulting from the oxidation of naphthalen-1-ol and N,N-dimethylbenzene-1,4-diamine by cytochrome oxidase enzymes (Harwig, 1967). Few minutes after immersion in tube containing the dye, the S. *corrugata* hairy root fragments (clone SCO-HR-FA8) were picked up, mounted on glass slides, and observed with the microscope.

3.1.2.5.2. Test of viability.

The test of viability was performed on roots using two fluorescents dyes for vital staining; Calcein AM (AM = acetoxymethyl ester) and Fluorescein Diacetate (FDA). Calcein-AM is taken up into the cell of hairy root; it is converted by esterases into calcein characterized by green color. This is capable of complexing calcium ions, resulting in a green fluorescence. Since only living cells possess sufficient esterases, only live cells fluoresce green after excitation. FDA is a non-fluorescent hydrophobic fluorescein derivative that can pass through the cell membrane whereupon intracellular esterases hydrolyze the diacetate group producing the highly fluorescent product fluorescein. The fluorescein molecules accumulate in cells that possess intact membranes so the green fluorescence can be used as a marker of cell viability. Cells that do not possess an intact cell membrane or an active metabolism may not accumulate the

fluorescent product and therefore do not exhibit green fluorescence. The solution of FDA stock solution was prepared by diluting FDA in acetone (5 mg/mL) and stored at -18°C. Immediately before staining, a sample of this solution was diluted 100 times with distilled water to make the final solution (50 µg/mL) and laid over the fresh material. A sample of the calcein AM stock solution 33.33 mg/mL (53.54 mM) was diluted 50 times with distilled water to obtain a solution of about 1 mM. Living hairy root was immersed in a drop of these solutions and kept for 15' (FDA) and 30' (calcein AM) at room temperature and in dark condition. The material was mounted on the microscopic glass slides and observed with the fluorescence microscopy (LEICA DM 4000 B with GFB filter cube: excitation range blue, excitation filter BP 470/40, dichromatic mirror 500, suppression filter BP525/50) and the pictures were taken with LEICA DFC 350 FX).

3.1.2.6. Growth kinetics of hairy root cultures in liquid MS medium.

The growth curve of fresh and dry weight was carried out on clone SCO-HR-FA8 during 42 days. The experiment was done in 250 mL glass vessel with transparent cap. 21 replications were prepared at T0, each one by inoculating 1 g of fresh hairy root accurately weighed into 50 mL of MS hormone free medium, incubated at dark condition under rotation at 120 rpm. At each interval of 7 days, three samples were randomly chosen and analyzed for fresh and dry weight (FW, DW) evaluation, pH and conductivity. Fresh hairy roots were filtered, blotted with tissue paper, and the fresh root weight was measured. For dry weight measurement, the fresh hairy roots were dried in oven at 60°C for 12 h and then weighed. The experiment was made in batch culture without change or fill the medium.

3.1.2.7. Hairy root growth into the bioreactor RITA®

3.1.2.7.1. Container evaluation for biomass production

The comparison of the growth of HR in different containers was performed comparing glass vessel (250 mL) with TIS bioreactor (RITA®) at the culture conditions reported in **Table 3.4**. The bioreactor closures were protected with parafilm. All the materials were cultured in dark. three independent biological replicates were used. The growth index (GI) was evaluated after 28 days culture. Fresh hairy roots were filtered, blotted with tissue paper, and the fresh root weight

was measured. For dry weight measurement, the fresh hairy roots were dried in oven at 60°C for 12 h and weighed. The growth index was calculated as follows:

$$GI = \frac{\text{Final weight- initial weight}}{\text{Initial weight}} \times 100$$

Table 3. 4 Conditions of investigation of container for biomass production

Containers	Initial fresh weight of HR (g)	Medium MS0 volume (mL)	Growth condition
Glass vessels	1	50	Rotation 120 rpm
Bioreactors (RITA®)	3	150	Immersion frequency: 3 Min flooding every 3 hours of stand-by periods

3.1.2.7.2. Effect of different media formulations on hairy root growth

To optimize the suitable culture medium for growth of hairy roots, three popular culture media formulations were tested (MS, B5 (Gamborg et al., 1968) and WPM (Lloyd & McCown, 1980)) at full concentration or at half strength: ½ MS, ½ B5 and ½ WPM. The composition of these medium is listed in (**Annex 1**). Hairy roots were cultivated in 0.5 liter TIS bioreactor RITA® containing 150 ml of the hormone-free liquid basal medium supplemented with 30 g/L sucrose. Cultivation was performed with the following immersion frequency: 3 min flooding every 3 hours of stand-by periods in dark at 23 ± 2°C for 30 days. 3 g of fresh weight biomass was used for each bioreactor. At the end of the culture period of 30 days, the growth rate of each culture was determined (FW and DW). For each treatment, four independent biological replicates were used.

3.1.2.7.3. Effect of initial sucrose concentration on hairy root growth

Three g of hairy roots were used as inoculum in 0.5 liter TIS bioreactor RITA® containing 150 ml of MS hormone-free liquid medium and the following addition of sucrose: 2%, 3% and 4% (i.e. 20, 30, 40 g/L), with the following immersion frequency: 3 Min flooding every 3 hours of stand-

by periods in dark at 23 ± 2°C. For each treatment, four independent biological replicates were used. After 30 days of culture, the biomass produced was harvested and analyzed.

3.1.2.7.4. Elicitation of hairy roots with different elicitors

Three elicitors were tested, each at three concentrations and in combination of them. They are representative of the major classes of elicitors for the induction of plant responses and the stimulation of secondary metabolite production in plant tissue cultures.

Abiotic Ag^+, signal molecule MJ, and biotic YE were applied in the hairy root elicitation. Ag^+ was prepared by dissolving $AgNO_3$ (ARDET) in distilled water (J-L. Zhao et al., 2010) to obtain the stock solution concentrated at 10 mg/mL. MJ (ALDRICH) was prepared by dilution of stock in 95% ethanol (Prakash et al., 2008) to have the mother solution of about 19.57 mg/mL. YE (Difco laboratorie) was obtained by the method of (Hahn et al., 1978). The pH of all elicitors was adjusted to value 5.7. The YE and $AgNO_3$ were sterilized by autoclaving at 1 atm and 121 °C for 20' and MJ by filtering through a microfilter (0.2 µm). All the stock solutions of elicitors were kept at 4°C.

To screen the best concentration of elicitor, Ag^+ was applied in HR with the concentration ranging of 15, 30 and 60 µM, MJ was added with concentrations of 50, 100 and 200 µM, and YE treatment was carried out with concentrations of 100, 200 and 400 mg/L. Moreover, the combination of YE (400 mg/L) with MJ and Ag^+ was also tested with the concentrations of 100 µM and 30 µM respectively (**Table 3.5**). The culture conditions were the same for the previous one (immersion frequencies with 3 min flooding and 3 h stand-by periods at 23 ± 2°C and in dark condition).

Table 3. 5 Elicitors and different concentration applied on hairy root culture

Elicitors	Concentrations		
Methyl jasmonate (MJ)	50 µM	100 µM	200 µM
Yeast extract (YE)	100 mg/L	200 mg/L	400 mg/L
Silver Nitrate (Ag)	15 µM	30 µM	60 µM
YE + Ag	400 mg/L + 30 µM		
YE + MJ	400 mg/L + 100 µM		
Control	-		

All the elicitors were added at days 21 in hairy root culture growing in 0.5 L bioreactor RITA® containing 150 mL of ½ WPM and 30 g/L of sucrose. Hairy roots were collected 7 days after the addition of elicitors. Fresh hairy roots were filtered, blotted with tissue paper, and the fresh root weight was measured. For dry weight measurement, fresh hairy roots were lyophilized and weighed. The medium pH and conductivity were also measured. For each treatment, three independent biological replicates were used.

3.1.2.7.5. Scale-up production of hairy root biomass

Hairy roots can be cultivated in bioreactors, which enable large scale and make the whole process more profitable. The biomass production was done following this sequences culture: first in Petri dish, second transfer in glass vessel and third transfer in 0.5-liter bioreactor. The transfer inside the bioreactor containing 150 mL MS0 was done when the fresh weight of hairy roots inside the glass vessel was around 5 g. The liquid medium MS0 was renewed completely every 10 to 15 days with fresh one. Three months after culture into the bioreactors, the total biomass in bioreactor was harvested and stored at -80˚C before lyophilization.

3.1.3. Phytochemical analysis

3.1.3.1. Extract preparation.

Salvia corrugata Shoots obtained after elicitation with MJ and light were dried by lyophilization and extracted exhaustively by maceration with methanol (three times) for 72 hours at room temperature. Following filtration through filter paper, the extracts were concentrated in vacuum at 60˚C using rotavapor until complete removal of solvent. Dry extracts were kept at 4˚C prior the analysis. The whole crude dry of all *S. corrugata* hairy roots clones were placed separately in container with methanol and allowed macerated to stand at room temperature with frequent agitation. The mixture was filtered every day for a period of at least 3 days. The solvent was eliminated through evaporation using Rotavapor.

On the dry extracts, there were investigated the fruticuline A and demethylfruticuline A production and accumulation. The clone SCO-HR-FA8 with the largest dry extract was carried out to purification techniques.

3.1.3.2. Qualitative quantitative analysis of Fruticuline A and demethylfruticuline A on S. corrugata in vitro biomass.

General experimental procedures

The analysis of the presence of fruticuline A and demethylfruticuline A in different *S. corrugata* extracts involved the application of common phytochemical screening assays, chromatographic techniques such as the rapid qualitative clean-up TLC, and the quantitative LC-MS/MS and HPLC-UV techniques.

Fruticuline A and demethylfruticuline A were obtained from the surface extract of fresh aerial parts of *S. corrugata* by Prof. Angela Bisio (Bisio et al., 1999) HPLC grade methanol, acetonitrile were obtained from VWR. The water was treated in a Milli-Q water purification system.

Thin-layer chromatographic

TLC analyses were carried out using protocol describe by (Bisio et al., 2008) with the TLC (20x20 cm) plates pre-coated with the silica gel Kieselgel 60 plates (Merck), 0.25 mm thick, with aluminum as support.

The plate was developed in the mobile phase (eluent) with chloroform, methanol and formic acid in the following proportions (10:0.5:0.1). This solvent system was chosen as it displayed good separation on a TLC plate. The sample was mounted on the plate 1 cm above its lower edge after extraction from the dry plant material and concentration with Rotavapor. The analyses were carried out in the glass chromatographic chambers leaving migrate the mobile phase from the lower plate edge until 90 mm distance. Prior to the development of the chromatograms, the chromatographic chamber was pre-saturated with the mobile phase for 20 min. The separated spots on TLC were identified under UV lights (254 nm and 366 nm). Then TLCs were sprayed with 50% H_2SO_4 followed by heating at 100 °C, allowing to the development of specific coloring that can give information about the nature of examined compound. After that, the spots were scanned.

Mass spectrometry

Mass spectra were acquired in positive ion mode on a Q-TOF spectrometer equipped with an electrospray ion source. LC-MS/MS analyses were carried out using a system consisting of a Shimadzu chromatography apparatus coupled with a QTRAP 6500+ System mass spectrometer equipped by a triple quadrupole.A mixed standard stock solution containing the reference compounds was prepared in methanol (1 mg/mL), and sequentially diluted with methanol to a series of appropriate concentrations (1, 5, 10, 15, 20 and 30 µg/mL). Then, the standard solutions were filtered through a 0.22 µm Millipore filter before injection. The calibration curves of these secondary metabolites were was obtained by preparing a series of concentrations of standard solutions (1, 5, 10, 15, 20 and 30 µg/mL) and then plotted the peak areas against the corresponding concentrations (**Fig. 3.3**).

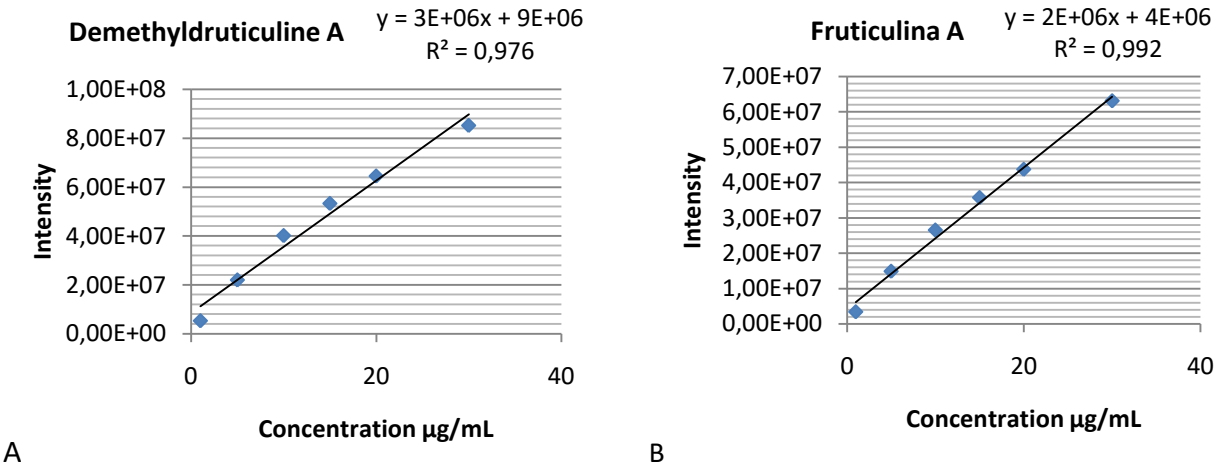


Figure 3.3. Calibrations curves of standard. (A) Demethylfruticuline A, (B) Fruticuline A

The solutions of *S. corrugata* shoot elicitated with MJ except the extract elicitated with 200 µM were prepared by dissolution of the dry extract in methanol to obtain the concentration of 1 mg/mL. All *S. corrugata* hairy root clones were prepared at the concentration of 15 mg/mL. Fruticuline A and demethylfruticuline A content in the culture medium was negligible and not determined. 3µL were injected onto a Kinetex 1.7 µm C18 100 Å, LC Column 50 x 2.1 mm. The mobile phase consisted of water (A) and CH_3CN (B) eluted with a linear gradient of A:B (45:55–0:100 over 15 min). All the samples prepared were determined the target peaks according to the retention time and mass/charge ratios (m/z) of those standards. Quantification was calculated by the linear calibration plots of the peak areas against the homologous concentration.

HPLC

The quantitative determination demethylfruticuline A into the *S. corrugata* shoot obtained after light elicitation was analyzed base on the HPLC protocol developed by (Bisio et al., 2016). The working standard solution was prepared from the stock solution by sequential dilutions with CH3CN 100% before each analysis. The concentrations of the working standard solution were 0.01–0.5 mg/mL. Working solutions of *S. corrugata* the shoots extract eliitted with light were prepared 1 mg/mL with CH3CN 100% immediately before each analysis. 20 µL were injected onto a Symmetry 300 C18 column, 4.6 × 250 mm ID, 5 µm particle size (Waters),

maintained at 25 °C. The mobile phase consisted of water (A) and CH3CN (B) eluted with a linear gradient of A:B (45:55–0:100 over 15 min) at a flow rate of 1.0 mL/min. Peak was detected at 220 nm.

3.1.3.3. Fractionation of *S. corrugata* hairy roots

The methanolic extracts of the SCO-HR-FA8 biomass (16.8 g) was fractionated by Si gel MPLC (Medium Pressure Liquid Chromatography). The methanolic extracts was mixed with a small quantity of Silica gel (Merck Kiesegel 60, 230–400 mesh) on the proportion (1:3 extract:Silica gel) dried at 60 °C in an oven, reduced to a fine powder and added to the top of the column. The column was eluted at 10 mL/min flow rate successively with n-hexane/$CHCl_3$/CH_3OH at concentrations varying from 100:0:0 to 0:0:100 (hexane 100% for 24 min, followed by chloroform 100% for 75 min, then chloroform:methanol (95:5) 51 min and finally by 100% methanol 21 min). The collection of these crude fractions were carried out in 30 mL tubes every 3 min to reach a total of 57 tubes. The fractions with similar spots (similar Rf values) on TLC were combined and concentrated to afford 16 main fractions (1-16). The fractions 6 were purified according to purified by semi-preparative RP HPLC (eluents A: H_2O, B: CH_3OH, gradient: B 5% at time 0 min, B 100% at time 61 min, B 100% at time 70 min).

3.1.4. Antibacterial activity

The antibacterial activity of methanolic extract of *S. corrugata* hairy root, their 16 semi-purified fractions obtained by its medium pressure liquid chromatography (MPLC) were assessed by determining Minimal Inhibitory Concentration (MIC) (µg/mL) value on Twenty two clinical strains, previously isolated from different clinical specimens and identified according to standard procedures (Murray et al., 1999). Twenty one were Gram-positive: three *S. aureus* (one isolated was oxacillin-resistant), three *S. epidermidis* (on strain was oxacillin-resistant and one oxacillin and Linezolid -resistant) and six other *Staphylococcus* strains *(S. saprophyticus, S. lugdunensis S. warneri, S. simulans, S. capitis, S. haemolyticus and S. hominis)* two *E. faecalis* (one vancomycin-resistant), two *E. faecium* (one vancomycin-resistant) and four other *Enterococci* (*E. gallinarum* vancomycin-resistant, *E. casselaflavus* vancomycin-resistant, *E. durans and E. avium*); and Gram-negative: *E. coli* UTI (*i.e.* obtained from a human urinary tract infection). One purified compound was assessed by determining MIC value on eight *Enterococci* strains: tow *E. faecalis* (one Vancomycin-resistant), two *E. faecium* (one Vancomycin-resistant), *E. durans, E. avium*, *E. gallinarum* Vancomycin-resistant *and E. casseliflavus* Vancomycin-resistant.

The MICs were determined following the microdilution procedure detailed by the clinical institute (Clinical and Laboratory Standards Institute, 2016). All the MICs were obtained in triplicate.

3.2. Materials and methods *Salvia tingitana* Etl.

3.2.1. *Salvia tingitana* shoot establisment

As starting material young leaves, apical shoots and nodes from mother plants of *S. tingitana* were selected and cut from the plant collection stored at the Agricultural Research Council Unit CREA-OF Sanremo (Italy); the seeds come from the personal collection of Dr. Claudio Cervelli.

3.2.1.1. In vivo root induction

21 shoot explants of about 5-10 cm from *in vivo* culture of mother plants of *S. tingitana* were immersed by the inferior part in a solution of K-IBA 3000 ppm for one min and transplanted into the substrate composed by peat:perlite (70%:30% V:V) and incubate in greenhouse under mist system (water spraying every 10''/ 40' from 9am to 5pm). Put under water spraying 10 seconds every 40 min from 9 am to 5 pm. After 40 days the rooting percentage was evaluated and plants were transferred into pot of 14 diameter containing commercial substrate.

3.2.1.2. Sterilization and *in vitro* shoot induction

To induce new shoots able to be used for *in vitro* culture establishment, the surface of apical shoot and node of *S. tingitana* explants from greenhouse were differently pre-treated and sterilized. The pre-treatment consists to wash explants with tap water for 15', with water containing detergent for 15' or dip into 70% (v/v) of ethanol for 60''. The sterilization was done with different concentration of commercial sodium hypochlorite solution (0.5, 1 and 2% of active chlorine) supplemented with 1% of Tween 20 for 10 – 15'; the explants were rinsed three times with sterile distilled water for 10' each (**Table 3.6**). The cut end part of the explants in contact with the pretreatment and sterilization solutions were cut and removed (about 5 mm). The obtained explants were then cultured on MS medium supplemented with BA 0.3 mg/L (Mascarello et al., 2006) at the following environmental conditions: 23 ± 2 °C, 16 h lighting photoperiod at 30 µE $m^{-2}s^{-1}$. For each treatment three glass vessel containing each four explants were considered. Massive micropropagation was then performed by 28-day subcultures in the same medium and at the same thermo-photo periodic conditions. After 4 weeks, the viability of the plant material and the contamination percentage was observed.

Table 3. 6 Design for pre-sterilization and sterilization for apical shoots and nodes of *S. tingitana* explants

Sequence	Pre-sterilization (time)			Sterilization agents		
-	Ethanol 70%	Soap water	Tap water	NaClO (%)	Tween 20 (%)	Time (min)
1	1'	-	-	0.5	1	10’
2	1'	-	-	1	1	10’
3	-	10’	-	0.5	1	15’
4	-	-	15’	0.5	1	15’
5	-	-	15’	1	1	15’
6	-	-	15’	2	1	15’

3.2.1.3. *In vitro* seed germination

Seeds were divided in two groups of 10 seeds each. The first group was pre-treated with ethanol 70 % for 1 min before sterilization. First and second groups were treated with 0.2 % (v/v) sodium hypochlorite solution (NaClO) supplemented with a few drops of Tween 20 for 10 min and finally rinsed four times with sterile water. The seeds were then inoculated on MS0 medium in dark and at 23 ± 2 °C for 8 days. After 15 days they were transferred in light condition. After 2 weeks, the root part of approximatively 3 mm from the root apex was cut with a sterile scalpel. The areal part of the seedling was transferred in MS solid medium supplemented with 1.33 µM of BA for the multiplication. Four weeks after, shoots of *S. tingitana* presented four nodes, and were cut in two parts with two nodes each and the shoots were transferred again in MS solid medium supplemented with 1,33 µM of BA for further multiplication

3.2.1.4. *In vitro* root induction

The shoot of about 2 cm with fully expanded leaves were cut and transferred to MS medium supplemented with auxin IBA or IAA at 1.33 µM each in 16 h of light at 30 µE $m^{-2}s^{-1}$ of photoperiod at 23 ± 2 °C. MS medium without auxin was used as control. After 30 days, the rooting activity was evaluated by recording rooting percentage, root number per shoot, root

length and frequency of callogenesis. The experiment was done with 6 explants for each treatment.

3.2.2. Establishment and analysis of callus from *Salvia tingitana* Etl.

3.2.2.1. Sterilization and establishment of callus induction

Leaf explants were taken from mother plant growing in the greenhouse. Leaves of second and third node were first washed with tap water for 15 min and then with soapy water for 15 min, followed by treatment with 1% of active chlorine supplemented with 1% of Tween 20 for 15 min. Explants were finally rinsed three times with sterile distilled water for 10 min each.

After sterilization, the leaves were cut along the midrib and the fragments (1 to 1.5 cm) were inoculated onto different culture media. All types of culture media consisted of agarized (8 g/l agar) MS medium added of 10 mg/L of ascorbic acid (Bisio et al., 2016; Kintzios et al., 1999) to reduce explants necrosis supplemented with different concentration of plant growth regulators: Kinetin (KIN) in combination with 2,4-dichlorophenoxyacetic acid (2,4-D) (in a factorial experiment see **table 3.7**. Media were adjusted to ph 5.7 ± 2 using NaOH or HCl, autoclaved at 121 °C and 1 atm for 20 min and poured into polystyrene Petri dishes, 90 mm diameter (25 ml of medium/dish). For each combination, three Petri dishes containing 6 leaf explants were considered and sealed with Parafilm. Cultural conditions were 23 ± 2 °C; 16 h of light at 30 µE $m^{-2}s^{-1}$ of photoperiod and also in dark condition. 4 weeks after, quantity and quality data were recorded. The frequency of callus induction was calculated according to the following formula:

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of explants producing callus}}{\text{No. of explants}} \times 100$$

After 4 weeks, a sample part of the callus was transferred to the respective culture medium without 2,4-D on the same conditions to explore the possibility to induce somatic embryogenesis.

Table 3. 7 Different combination of growth regulators (mg/L) used to induce callus from leaf explants of *S. tingitana.*

KIN 2,4-D	0	0.5	1	5
0	0; 0	0; 0.5	0; 1	0; 5
0.1	0.1; 0	0.1; 0.5	0.1; 1	0.1; 5
0.5	0.5; 0	0.5; 0.5	0.5; 1	0.5; 5
1	1; 0	1; 0.5	1; 1	1; 5

3.2.2.2. Callus viability

The test of viability was performed using Fluorescein Diacetate (FDA). The solution of FDA stock solution was prepared by diluting FDA in acetone (5 mg/mL) and stored at -18°C. Immediately before staining, a sample of this solution was diluted 100 times with distilled water to make the final solution (50 µg/mL) and laid over the fresh material. Living callus was immersed in a drop of this solution for 30' in dark condition. The material was mounted on the microscopic glass slides and observed with the fluorescence microscopy.

3.2.2.3. Influence of growth regulators on callus biomass production.

3.2.2.3.1. Effect of different concentrations of KIN and 2,4-D.

The experiment was done to determine the best concentration of growth regulators for the biomass production. Three concentrations of KIN (0.1; 0.5 and 1 mg/L) in combination with 2,4-D (0.5 and 1 mg/L) and medium without hormone MS0 (**Table 3.8**) were used. 6 Petri dishes were prepared for each combination and the fresh and dry weights were evaluated after 35 days of culture in dark condition and at temperature of 23˚C ± 2.

Table 3. 8 Different combination of growth regulators (mg/L) used to evaluate callus growth biomass.

KIN 2,4-D	0	0.5	1
0	0: 0	-	-
0.1	-	0.1; 0.5	0.1; 1
0.5	-	0.5; 0.5	0.5; 1
1	-	1; 0.5	1; 1

3.2.2.3.2. Effect of PGRs types

The callus produced on medium supplemented with KIN 0.5 mg/L, 2,4-D 0.5 mg/L and 10 mg/L of ascorbic acid was used to determine the best growth regulators for further experiment the biomass production. In this experiment, the concentrations were reported in molarity in order to compare growth regulators. Equimolar level of different kind of cytokinin at 2.32 µM (Kinetin, Benzyl adenine, and meta-Topolin) were combined with 2,4-D 4.52 µM supplemented with 10 mg/L of ascorbic acid. In other to find the best auxin, the Kinetin 2.32 µM was combined with equimolar concentration of different auxin 2,4-D or NAA at 4.52 µM, supplemented with 10 mg/L of ascorbic acid. 4 Petri-dishes were prepared for each combination. The fresh and dry weights were evaluated after 35 days of culture in dark condition and at temperature of 23˚C ± 2.

3.2.2.4. Growth kinetic

I order to develop a *S. tingitana* callus growth curve; pieces of 2 g of fresh callus were inoculated onto 25 mL solid MC medium in dark and at 23 ± 2 °C. 18 Petri dishes were prepared. Every one-week interval, up to five weeks three Petri-dishes were randomly chosen and analyzed for fresh weight and dry weight.

3.2.2.5. Callus biomass production

S. tingitana callus was then cultured onto Petri-dishes containing 25 mL of MS medium supplemented with KIN 2.32 µM; 2,4-D 4.52 µM and 10 mg/L of ascorbic acid named MC medium. The medium was changed every 5 weeks during 14 months to reach a large biomass amount. The final biomass yield was obtained by culturing 80 Petri-dish containing the same medium composition and initially charged with fresh callus about 3.55 g of into 25 ml of medium. The cultures were incubated in the growth chamber at 23 ± 2 °C in dark for 5 weeks. The total biomass was harvested and the fresh and dry weights were determined.

3.2.2.6. Elicitation of *Salvia tingitana* callus

3.2.2.6.1. Elicitation with methyl jasmonate

5 g of callus grown onto MC medium was transferred after 21 days onto different media with the same growth regulators as previous added with concentrations of MJ (50, 100 and 200 µM). Ten Petri-dishes were prepared for each combination. The cultures were incubated in dark condition for 2 weeks and after that, they were harvested and the fresh and dry weights were analyzed.

3.2.2.6.2. Elicitation with light

In order to determine the effect of light and dark on callus growth production, 3.55 g of fresh callus were cultured in dark condition onto 25 mL of MC medium; 12 Petri-dishes were prepared. On day 21, 6 Petri were transferred in light condition (16 h lighting photoperiod at 30 µE $m^{-2}s^{-1}$), and the others were maintained in dark. On the day 36, the callus was harvested and the fresh and dry weights were analyzed.

3.2.3. Phytochemical analysis

Callus produced were dried by lyophilization and extracted exhaustively by maceration with methanol (three times) for 72 hours at room temperature. Following filtration, the extracts were concentrated in vacuum at 60˚C using Rotavapor until complete removal of solvent. Dry extracts were kept at 4˚C prior the analysis. The TLC analysis on different extract was investigated according to TLC protocol present on **section 3.1.4.2.**

The methanolic extracts of large *S. tingitana* callus biomass was semi-purified according to the protocol explained on **section 3.1.3.4** . The fractions were collected in 30 mL tube and similar spots on TLC were combined to yield 13 main fractions (1-13)

3.2.4. Antibacterial activity

The antibacterial activity of methanolic extract of *S. tingitana* callus and their 13 semi-purified fractions obtained by MPLC were assessed by determining Minimal Inhibitory Concentration (MIC) (µg/mL) value on *E. coli* UTI (*i.e.* obtained from a human urinary tract infection), two *staphylococci* strains *(S. aureus* and *S. epidermidis*) and *Enterococci* strains (*faecalis* and *E. faecium)* clinical strains, isolated from different clinical specimens and identified according to standard procedures (Murray et al., 1999). The MICs were determined following the microdilution procedure detailed by the clinical institute (Clinical and Laboratory Standards Institute, 2016). All the MICs were obtained in triplicate.

3.3. Statistical analysis

Data were analyzed using the R environment (R Core Group, 2018). All experiments were conducted using a completely randomized design. The analysis of variance was conducted using the aov( ) function [from the package *stats*]. Before the analysis, data distributions were checked for normality using the shapiro.test( ) function. When needed, data were transformed in order to fulfill the normality requirement. The influence of the initial plant weight on the final weight was checked by means of a correlation test using of the cor.test( ) function. After the ANOVA analysis, treatment levels were compared for significant differences using Tukey's test ($P< 0.05$) with the function TukeyHSD( ). The data are presented as means ± standard deviation or standard error.

CHAPTER 4 RESULTS AND DISCUSSIONS

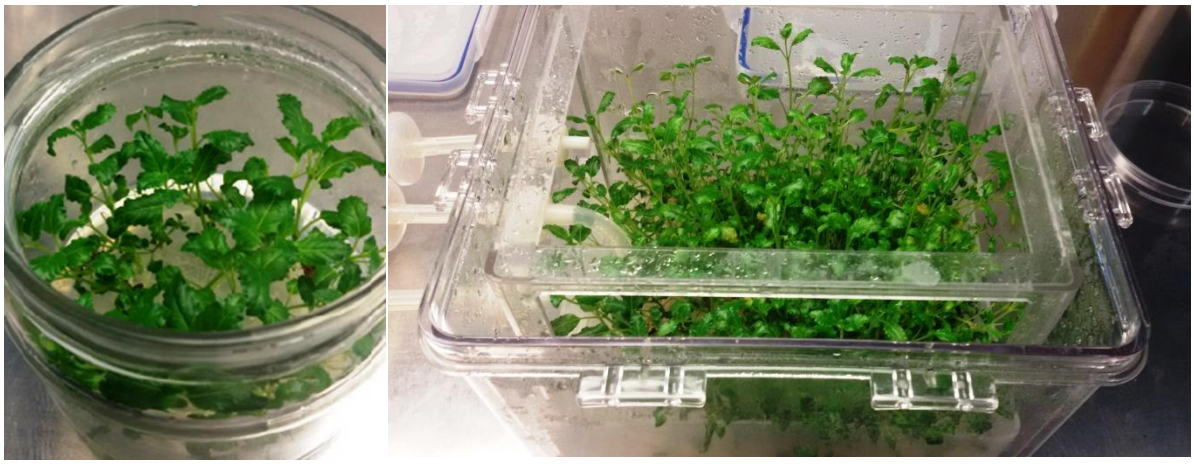
4.1. Results

4.1.1. Results of *Salvia corrugata*

4.1.1.1. *Salvia corrugata* shoot in vitro development and elicitation

4.1.1.1.1. In vitro shoot culture establishment

Sterile and alive shoots of *S. corrugata* were obtained after sterilization with active chlorine and culture into MS0 (**Fig. 4.1)**. The shoots were able to grow and multiply at low rate in total absence of growth regulators. However, reached multiplication rates 3 or 4 times higher when cultivated with 0.3 mg/L BA and more than 4 when cultured into the TIS Plantform bioreactor. It was observed that shoot developed root when it was culture in MS medium in absence of BA. This material represents the starting material for all the following *in vitro* experiment about the shoot elicitation and hairy root transformation.



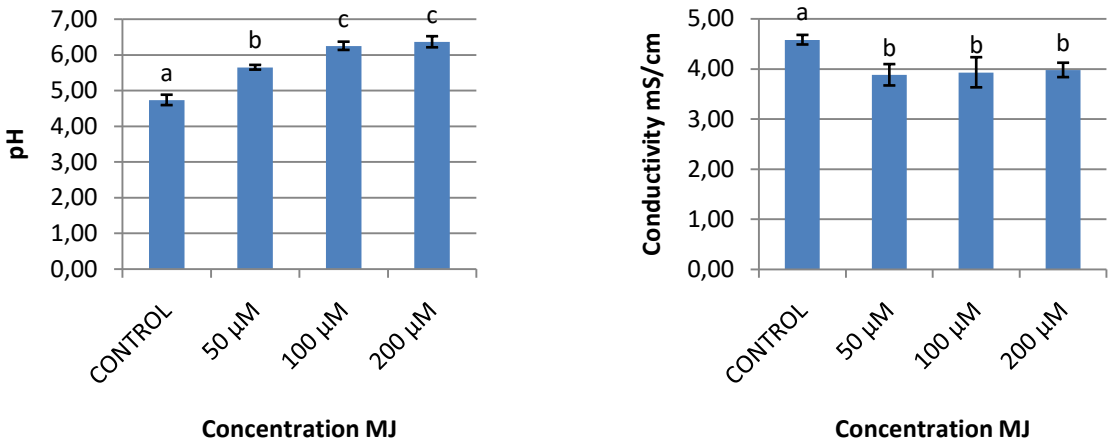
(A) (B)

Figure 4. 1 In vitro culture of *S. corrugata* shoots. (A) onto solid MS0 solid medium, (B) onto TIS Plantform containing MS supplemented with BA.

4.1.1.1.2. Shoot elicitation in bioreactor with methyl jasmonate

In **Fig. 4.2** the values of pH and conductivity recorded in the vessels treated with MJ (50, 100 and 200 µM) at day 21 were shown. The value of pH is statically the highest (6.36 ± 0.16) at 200 µM and (6.25 ± 0.12) at 100 µM. the non-treated shoots presented the lowest pH (4.74 ± 0.16). The conductivity presented the highest value (4.6 ± 0.1 mS/cm) in the non-treated shoots. Not

significant differences were obtained among all the treated samples**.** The fresh and dry weight of plant material evaluated at day 28 did not show any statistical differences among all the treatments. The fresh and dry weights were reported **Table 4.1**, and the quality of shoots was shown in **Fig 4.3**.



A B

Figure 4. 2 pH and conductivity in the media of *S. corrugata* shoot culture in bioreactor after elicitation with MJ of 3 days of treatment. Values are means ± standard deviation n= 3; different letters identify values which differ at *p ≤ 0.05*.

18 days before elicitation

28 days 50 µM 28 days 100 µM 28 days 200 µM

Figure 4. 3 Shoot culture in the TIS bioreactors with different concentrations of MJ.

Table 4. 1 Fresh and dry weight after elicitation with different concentrations of MJ evaluated at day 28. Values are means ± standard deviation.

Treatment	FW ± SD (g)	DW ± SD
Control	9.98 ± 1.48	0.89 ± 0.16
50 µM	10.35 ± 0.78	1.05 ± 0.16
100 µM	9.63 ± 1.74	1.03 ± 0.28
200 µM	10.03 ± 1.14	1.05 ± 014

4.1.1.1.3. Determination of the content of demethylfruticuline A and fruticuline A in the in shoot elicitated with methyl jasmonate

The quantitative analysis of selected secondary metabolite (Fruticuline A and demethylfruticuline A) produced and accumulated into *S. corrugata* shoots elicitated with different concentration of MJ were detected. The MS/MS detection parameters of 2 standards (pure fruticuline A and demethylfruticuline A) are shown in **Table 4.2.**

Table 4. 2 Parameters of the two target compounds studied.

Compounds	Rt (min)	MW	MRM transitions	DP (V)	EP (V)	CE (V)	CXP (eV)	Ion mode
Demethylfruticuline A	2.96	310.34	241.0>251.1>222.9	97	14	28	26	ES+
Fruticuline A	3.63	324.37	254.1>233	89	11	32	13	ES+

Fruticuline A and demethylfruticuline A content under MJ treatment decreased compared to the control (**Table 4.3**). Representative chromatograms of the LC-MS/MS (MRM) profile of fruticuline A and demthylfruticuline A are reported in **Annex 5**. Fruticuline A decreased more than 2-fold and 3.4-fold at the concentration of 50 µM and 100 µM respectively compared to the control. The amount of demethylfruticuline A decreased about 3-fold and 6-fold at the concentration of 50 µM and 100 µM respectively compared to the control.

Table 4. 3 Analysis of fruticuline A and demethylfruticuline A after elicitation of *S. corrugata* shoots with MJ. Values are means ± standard deviation n= 3; different letters identify values which differ at $p \leq 0.05$.

Treatment	Fruticuline A (mg/g DW)	Demethylfruticuline A (mg/g DW)
Control	5.46 ± 0.13 a	2.59 ± 0.08 a
MJ 50 µM	2.69 ± 0.02 b	0.83 ± 0.08 b
MJ 100 µM	1.59 ± 0.02 c	0.43 ± 0.01 c
MJ 200 µM	n.a	n.a

n.a not analyzed

The above result clearly shows that, MJ inhibit the biosynthesis of fruticuline A and demethylfruticuline A in *S. corrugata* shoot culture.

In general, elicitation could have a negative effect on plant growth (Yukimune et al., 2000). In our study, the short time exposure of *S. corrugata* shoots to MJ for 3 days combined to the renewal of medium with MS0 allowed the reduction or suppression of negative effect of MJ on the shoot growth that remains comparable to the control. The use of MJ as elicitor to improve the increase the production and accumulation of secondary metabolites into the *S. corrugata* shoots showed an inhibitory effect on fruticuline A and demethylfruticuline A production and accumulation.

4.1.1.1.4. Shoot elicitation in glass vessel with light

The shoots cultured in dark condition presented long internodes and small leaves of green yellowish color, while the shoots exposed to light presented short internodes and the leaves were more large and showed an intense green color that increases gradually along with light intensity (**Fig. 4.4**). The analysis of fresh and dry weight of *S. corrugata* shoots exposed to different light intensities, showed the lowest values in dark condition (1.6 ± 0.2 g and 0.2 ± 0.01 g respectively). The light presence at any level increases both fresh and dry weight in all treatment in a significative way respect to the control. The fresh and dry biomass production

were reported after treatment with light intensity 30, 45 80 and 100 µE.m^{-2}.s^{-1} with values of 4.7 ± 0.6 g, 4.46 ± 7 g, 5.2 ± 0.1 g and 5.3 ± 0.7 g respectively for the fresh weight; and 0.58 ± 0.07 g, 0.61 ± 0.8 g, 0.62 ± 0.7, and 0.73 ± 0.11 g respectively for the dry weight (**Fig. 4.5**).

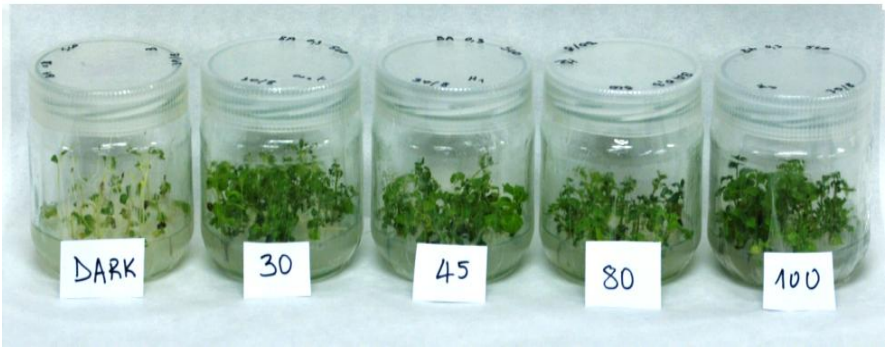


Figure 4. 4 Aspect of *S. corrugata* shoots 21 days after exposition on different light intensities (µE.m^{-2}.s^{-1}).

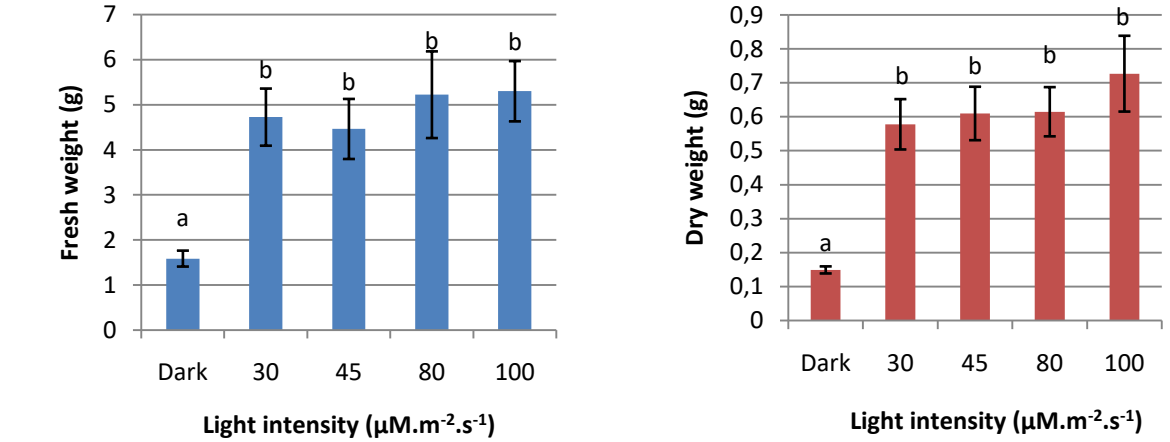


Figure 4. 5 Fresh (A) and dry (B) weight of *S. corrugata* shoots exposed at different light intensities for 21 days. Values are means ± standard deviation n= 4; different letters identify values which differ at *p ≤ 0.05*.

4.1.1.1.5. Demethylfruticuline A analysis on shoot elicitated with light

The qualitative TLC analysis of different methanolic extracts of *S. corrugata* shoots elicited with light allowed to note the presence of band relative to demethylfruticuline A mostly at light than at dark condition (**Fig. 4.6**).

For quantitative determination of demethylfruticuline A in the methanolic extracts of vitro shoots culture elicitated with light, an HPLC method was established using a linear gradient elution of water and CH_3CN at a flow rate of 1.0 mL/min. Representative chromatograms of demethylfruticuline A are reported in **Annex 6.** It was demonstrated that at any experimental condition, it is possible to induce the production demethylfruticuline A in *in vitro* grown shoots.

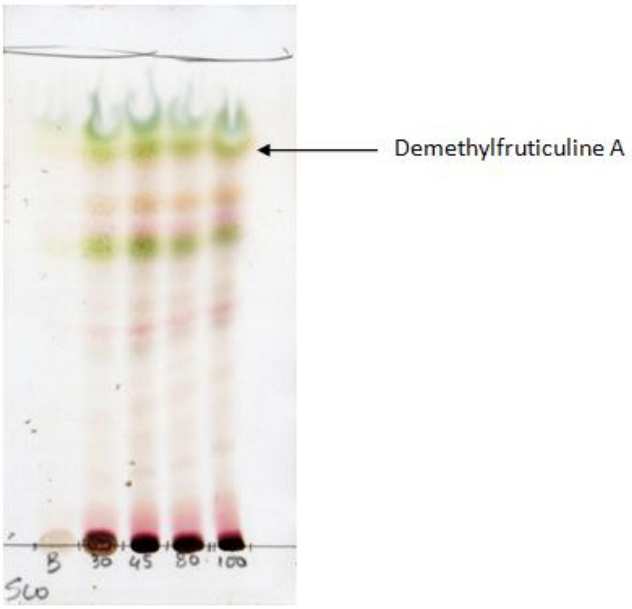


Figure 4. 6 TLC comparisons of different shoot extracts of *S. corrugata* exposed to different light intensities showing demethylfruticuline A.

Table 4. 4 Production of demethylfruticuline A on *S. corrugata* shoot elicitated with light.

	Demethylfruticuline A	
Light intensities ($\mu E.m^{-2}.s^{-1}$)	Means (mg/g DW)	S.D
0	0.34	0.07
30	0.21	0.05
45	0.21	0.05
80	0.47	0.15
100	n.a	n.a

4.1.1.2. Establishment and analysis of hairy roots from *Salvia corrugata* Vahl.

4.1.1.2.1. Induction and establishment of hairy root culture.

After the infection of *S. corrugata* explants with *A. rhizogenes* strains ATCC 15834 and LBA 9402, the putative roots were observed at the wounded sites of the explants after 10 to 14 days after inoculation (**Fig. 4.7)**. The transformation efficiency was influenced by the type of the bacterial strain. After 30 days sub-culture, the highest frequency of hairy root induction (75%) was achieved on explants infected with strain ATCC 15834, while the hypervirulent strain LBA 9402 induced putative roots in 38% of treated fragments. On the untreated explants (control), it was observed 14.3% of root development (**Table 4.5**).

(A)  **(B)** 

Figure 4. 7 Hairy roots of *S. corrugata* induced at the wounded sites of the explants 14 days after treatment by *A. rhizogenes* strain. (A) ATCC 15834 and (B) LBA 9402.

Table 4. 5 Induction of hairy roots by different strains of *A. rhizogenes* 30 days after inoculation.

***Agrobacterium rhizogenes* strain**	**Number of explants**	**Percentage of explants producing roots (%)**
ATCC 15834	16	12 (75%)
LBA 9402	21	8 (38.1%)
Control	21	3 (14.3%)

4.1.1.2.2. Clones selection and maintenance of hairy roots

Different clones were obtained by excision and isolation of putative roots from the mother leave explants. From *A. rhizogenes* strain ATCC 15834, 26 clones were isolated, 8 clones from strain LBA 9402 and 1 from control and named (**Table 4.6**). These roots were grown separately on 3 cm Petri-dish containing 3 mL solid hormone-free MS medium supplemented with cefotaxime for 30 days in order to investigate the daily increases of length and branch index. Among the clones, hairy root SCO-HR-FA8 and SCO-HR-FA13 from wild type *A. rhizogenes* ATCC 15834 showed a daily increase in length of 1 mm and branching of 0.20 and 0.13 respectively (**Table 4.7**), and clone SCO-HR-FL7 from strain LBA 9402 (**Table 4.8**) showed the highest daily increases in length of 0.70 mm and branching of 0.33. However, the root clone from the control explants decreased the growth once excised from the mother explant. In fact, transferred to the new medium had a daily increase of 0.07 mm reaching 2 mm in a month and had no formation of branches (**table 4.9**). However, not all isolated HR clones of *S. corrugata* displayed both a hairy root phenotype and a vigorous growth. Indeed, during this period, some of the hairy root clones stopped their growth and eventually died.

Table 4. 6 Root selection after 30 days of induction from *S. corrugata* leaves explants.

***Agrobacterium rhizogenes* strain**	**Total number of roots**	**Mean number of roots per explants**	**Number of root excised and transferred**
ATCC 15834	33	2.7	26
LBA 9402	21	2.6	8
Control (-)	5	1.7	1

Table 4. 7 Daily increases of length of the principal root and branching index (number of branches in 1 month/30) of different clones from wild type ATCC 15834 (1).

A. rhizogenes ATCC 15834	Increase (30 days later)		Daily increase		Missing of geotropism
clones	branch number	Length (mm)	branching index	Length (mm)	
SCO-HR-FA1	2	20	0.07	0.67	++
SCO-HR-FA2	1	30	0.03	1.00	+++
SCO-HR-FA3 *	0	0	0.00	0.00	-
SCO-HR-FA4 *	0	0	0.00	0.00	-
SCO-HR-FA5	1	30	0.03	1.00	-
SCO-HR-FA6 *	0	0	0.00	0.00	-
SCO-HR-FA7 *	0	0	0.00	0.00	-
SCO-HR-FA8 **	**6**	**30**	**0.20**	**1.00**	**+++**
SCO-HR-FA9	1	1	0.03	0.03	-
SCO-HR-FA10 *	0	0	0.00	0.00	-
SCO-HR-FA11	0	0	0.00	0.00	-
SCO-HR-FA12	0	10	0.00	0.33	+
SCO-HR-FA13 **	**4**	**30**	**0.13**	**1.00**	**+++**
SCO-HR-FA14 *	0	0	0.00	0.00	+
SCO-HR-FA15	0	15	0.00	0.50	+
SCO-HR-FA16	0	2	0.00	0.07	-
SCO-HR-FA17 *	0	0	0.00	0.00	-

Table 4. 7 Daily increases of length of the principal root and branching index (number of branches in 1 month/30) of different clones from wild type ATCC 15834 (2).

A. rhizogenes ATCC 15834	Increase (30 days later)		Daily increase		Missing of geotropism
clones	branch number	Length (mm)	branching index	Length (mm)	
SCO-HR-FA18 *	0	0	0.00	0.00	-
SCO-HR-FA19	3	29	0.10	0.97	++
SCO-HR-FA20 *	0	0	0.00	0.00	-
SCO-HR-FA21	2	16	0.07	0.53	+
SCO-HR-FA22	0	1	0.00	0.03	-
SCO-HR-FA23 *	0	0	0.00	0.00	-
SCO-HR-FA24	1	1	0.03	0.03	-
SCO-HR-FA25	0	12	0.00	0.40	+
SCO-HR-FA26	1	18	0.03	0.60	+

* Died clones after 1 month

** Selected clones

Table 4. 8 Daily increases of length of the principal root and branching index (number of branches in 1 month/30) of different clones from strain LBA 9402.

A.rhizogenes LBA 9402	Increase (30 days later)		Dailyincrease		Missing of geotropism
clones	Branch number	Length (mm)	Branching index	Length (mm)	
SCO-HR-FL1	0	3	0.00	0.10	-
SCO-HR-FL2	1	28	0.03	0.93	+
SCO-HR-FL3	0	1	0.00	0.03	-
SCO-HR-FL4	8	21	0.27	0.70	+
SCO-HR-FL5	0	10	0.00	0.33	+
SCO-HR-FL-6	1	24	0.03	0.80	+++
SCO-HR-FL7 **	**10**	**21**	**0.33**	**0.70**	**+++**
SCO-HR-FL-8 *	0	0	0.00	0.00	-

* Died clone after 1 month

** Selected clone

Table 4. 9 Daily increases of length principal root and branching index (number of branches in 1 month/30) of clone from control.

Control	Increase (30 days later)		Dailyincrease		Missing of geotropism
clone	Branch number	Length (mm)	Branching index	Length (mm)	
CTRL1	0	2	0.00	0.07	-

The selected clones were sub-cultured onto MS0 containing cefotaxime reduced to 50 mg/L for the clones SCO-HR-FA8 and SCO-HR-FA13, and to 300 mg/L for the clone SCO-HR-FL7 for another month. At this moment, cefotaxime was completely removed from the medium and hairy root not presented the sign of bacterial growth. The putative hairy root clones were cultured for one other month onto MS0 without cefotaxime to be sure of the absence of bacteria. The clones were transferred for other two months onto solid medium (**Fig. 4.8** A). These clones showed a very good growth once transferred and

subcultured into MS0 liquid medium (10 mL) under rotation of 60 rpm for on interval of 10 to 15 days. All of them grew very well in the liquid medium (**Fig. 4.8** B). Based on these result, the roots were multiplied in liquid medium into other glass vessels (**Fig. 4.8** C)

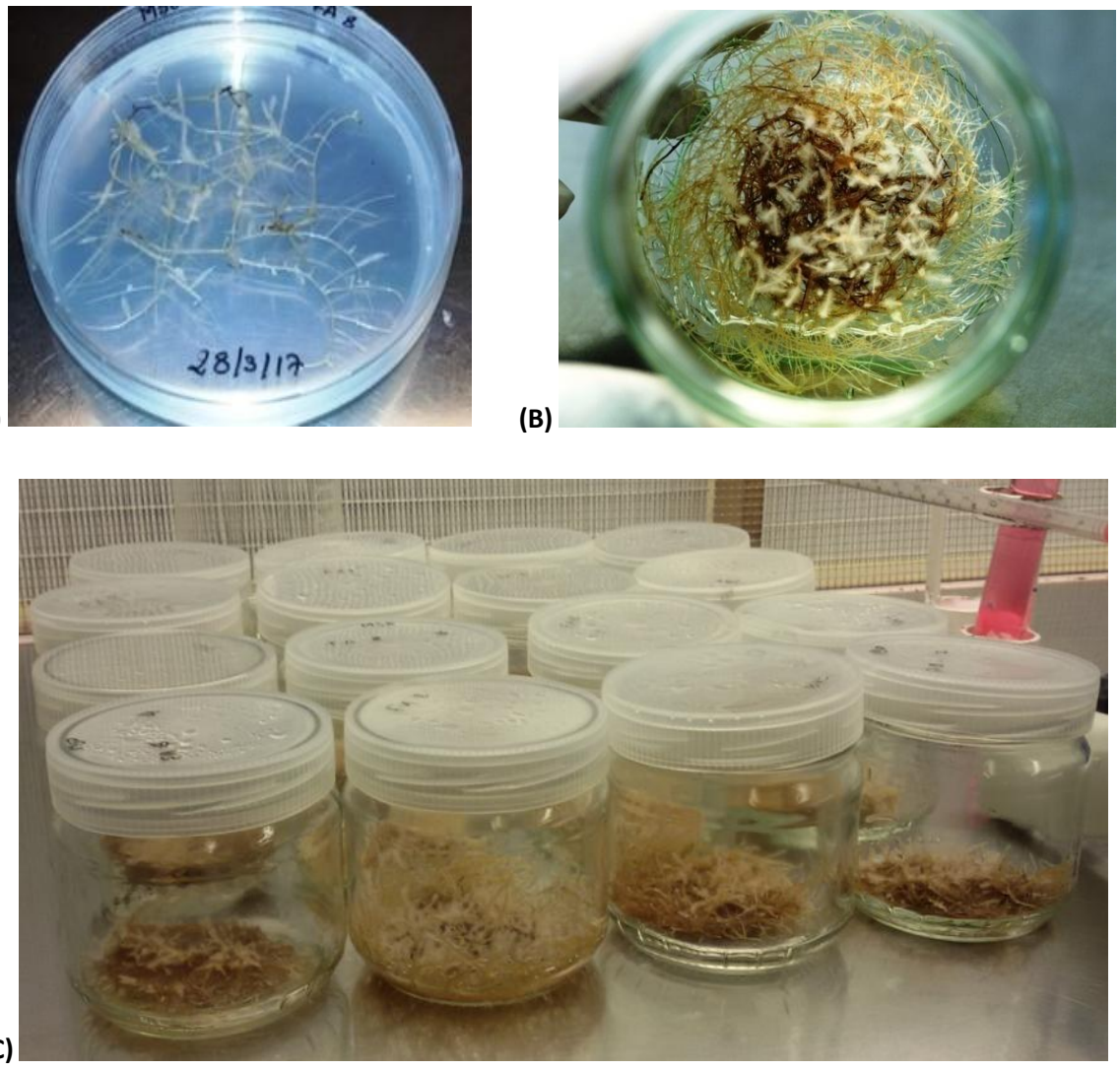


Figure 4. 8 Clone SCO-HR-FA8 putative hairy roots growth on solid hormone-free MS0 medium 90 days after transformation by ATCC 15834 (A); culture in liquid medium (B); multiplication into the glass vessel (C).

4.1.1.2.3. Detection of Ri T-DNA integration

4.1.1.2.3.1. DNA extraction

From the fresh roots of the three selected clones SCO-HR-FA8, SCO-HR-FA13, SCO-HR-FL7 cultured into Petri-dish containing MS0 medium and the not transformed control roots plant genomic DNA was extracted by Dneasy Plant Kit (Qiagen®) and the quantification was reported in (**Table 4.10**). Positive control was represented by plasmid DNA of *Agrobacterium rhizogenes* strain ATCC 15835 that was carried out from plasmid according to the Klimyuk method.

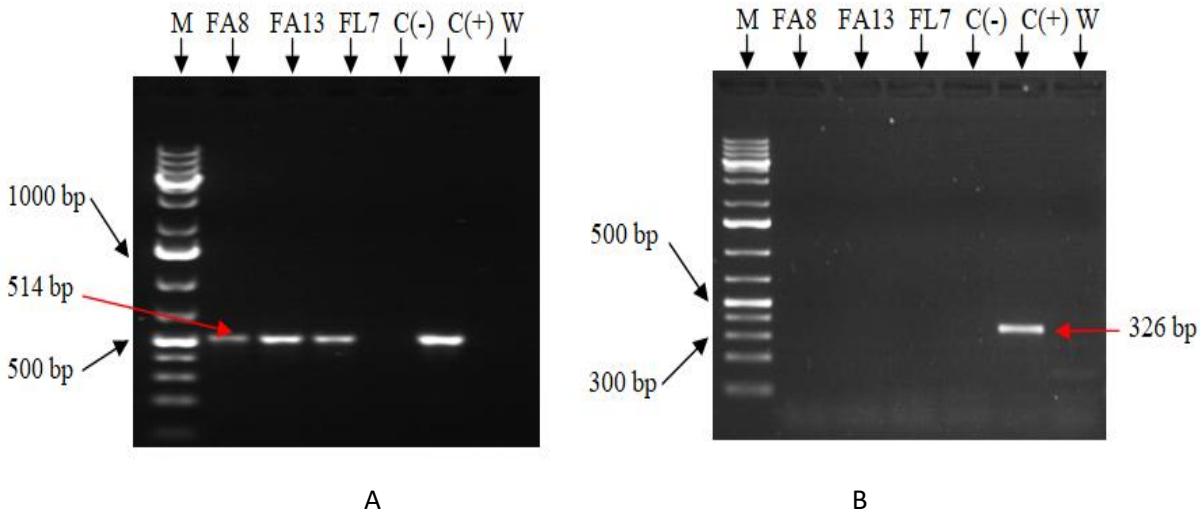
Table 4. 10 *S. corrugata* DNA quantification from different hairy root clones and non transformed root.

Clones	Fresh weight of root (mg)	Nucleic Acid (DNA) Conc. (ng/µl)
SCO-HR-FA8	83	30.2
SCO-HR-FA13	62	23.7
SCO-HR-FL7	106.2	26.2
Control (-)	100	16.3

4.1.1.2.3.2. PCR analysis and electrophoresis

In order to assess the genetic status of the hairy root and confirm the integration of T-DNA roots from *A. rhizogenes* into the hairy root genome, PCR was used to target the *A. rhizogenes rolC* gene (fragment of 514 bp) located on independent T-DNAs (TL-DNA) of Ri plasmid. Three clones (SCO-HR-FA8, SCO-HR-FA13 and SCO-HR-FL7) were detected positive to *rolC* gene as well as the positive control. While not-transformed root and water solution, supposed as negative controls, were detected negative (**Fig. 4.9** A) indicating that hairy root lines were successfully transformed. The absence of bacterial contamination was confirmed by PCR amplification of 326 bp fragment of the *virC1* gene. The amplification band of the *virC1* gene was detected positively only in the *A. rhizogenes* sample (positive control) while all the hairy root clones, non-transformed root and the solution without DNA were detected negative to *virC1* gene (**Fig. 4.9** B)**.** PCR amplification of 514 bp fragment of *rolC* gene and 326 bp fragment of the *virC* gene

showed that all clones selected contained the transgene into their genome and they were not contaminated.



A B

Figure 4. 9 Agarose gel electrophoresis of PCR amplification of genes in transformed roots. (A) The expected 514 bp *rolC* fragment; (B) the expected 326 bp *virC1* fragment. M= marker 1kb plus; FA8, FA13 and FA25: respectively clone of hairy root SCO-HR-FA8, SCO-HR-FA13 and SCO-HR-FL7; C(-); not-transformed root (negative control); C(+) plasmid DNA of *A. rhizogenes* ATCC 15834 (positive control); W solution without DNA.

Based on the typical morphologies (**Table 4.7** and **4.8)** and the confirmation at the molecular level, it could be concluded that the selected *S. corrugata* clones SCO-HR-FA8, SCO-HR-FA13 and SCO-HR-FL7 were hairy roots

4.1.1.2.4. Histochemical analysis of *Salvia corrugata* hairy root.

4.1.1.2.4.1. Terpene staining

The Nadi reagent, specific for terpenes, displayed a strong positive response. As reported in **Fig. 4.10**, it was found in hairs of *S. corrugata* hairy root the presence of many scattered blue and purple droplets justifying the presence of lipid and terpenes respectively. However, the purple droplets can also represent lipid droplets in which terpenes are dissolved. We could also observe in this figure the predominant localization of lipids along the hair wall.

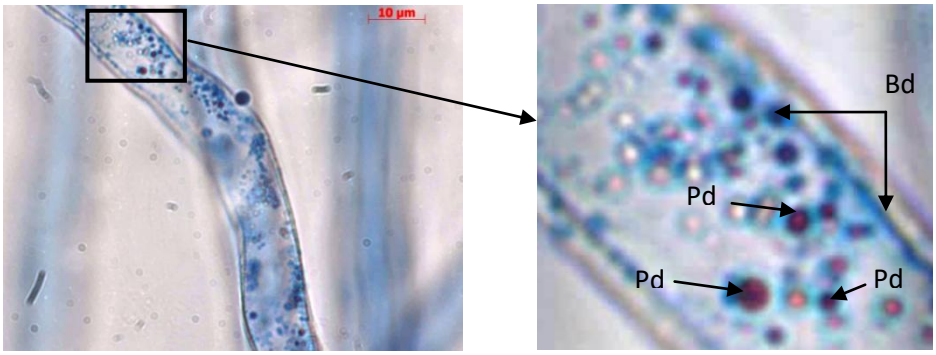


Figure 4. 10 Hairs staining of *S. corrugata* hairy root with Nadi reagent showing the presence of terpenes (Purple) and lipids (Blue) droplets. (60×).

4.1.1.2.4.2. Test of viability.

After calcein staining, at the top of the root, it was observed a strong intensity of green fluorescence that tends to decrease along the root structure (**Fig. 4.11** A). However, the lateral hairs were slightly reactive to calcein-AM and it was possible to observe them only on Bright-field (BF) option (**Fig. 4.1**1 B). The FDA stain showed very well the hairs both on florescent light and on Bright-field (BF) option (**Fig. 4.11** C and D) inside the root non-viable cells pale stained or gave any fluorescence (**Fig. 4.11** C). This observation makes it possible to highlight that the diameter of the root is about 200 µm.

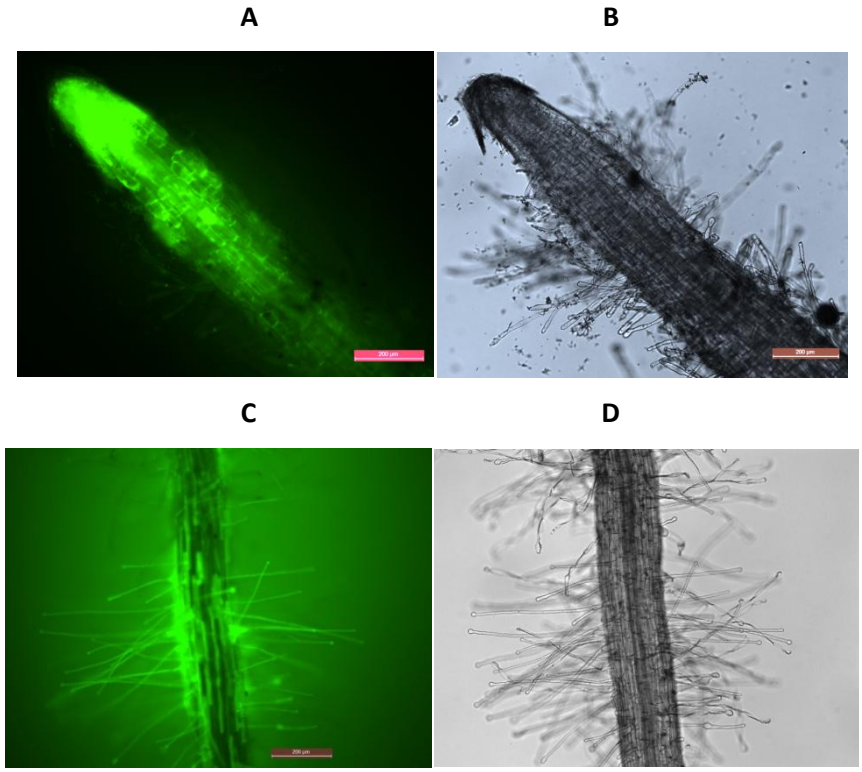
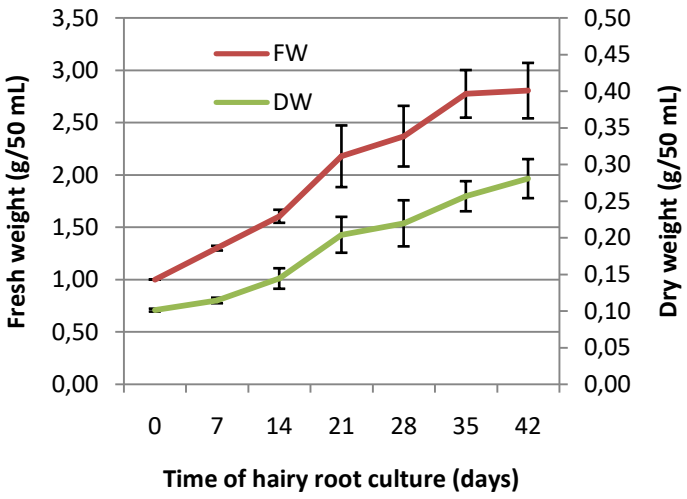


Figure 4. 11 Viability of hairy root with fluorescent dyes (A and B calcein AM; C and D FDA) with fluorescence microscopy. (10×).

4.1.1.2.5. Growth kinetics of hairy root cultures in liquid MS medium

Hairy roots cultured in MS0 medium were sampled with an interval of 7 days, and a typical sigmoid curve was determined after the growth kinetics assessment based on the fresh and dry weights evaluation (**Fig. 4.12**). The fresh hairy roots showed latent phase (lag phase) in the first weeks and then grew gradually and a growth logarithmic phase (2-5 weeks) appeared owing to the rapid cell division and excess of nutrients (exponential phase), yet a coming plateau phase was present at the end of the curve (week 5 to 6). The **fig. 4.13** showed the growth correlation between the fresh and dry weight during 6 weeks. After 6-week-long culture, the hairy roots showed a dense net and the highest biomass production. At this period, about 2.81 ± 0.27 g FW and 0.28 ± 0.03 g DW of hairy roots were harvested presenting the dry to fresh mass ratio of 10.00 % indicated a high dry matter accumulation of the hairy root cultures. However, hairy roots at this period showed at the biomass center a dark brown color that lacked viability and at the external part a clear and vital biomass (**Fig. 4.14**). The medium remained in the glass vessel presented a turbid a light brown color. Thus analyzing the growth curve, the most promising interval to perform elicitation is in the middle of the log phase (days 21-28)21-day-old hairy roots at the intermediate growth stage were favorable for elicitation.



A

Figure 4. 12 Growth curve of *S. corrugata* hairy root (clone SCO-HR-FA8) in MS0 liquid medium in dark at 120 rpm. FW (fresh weight) and DW (dry weight); 1 g of fresh hairy root was cultured in 250 mL glass vessels with 50 mL of MS0 liquid medium for 6 weeks and sampled with an interval of one week.

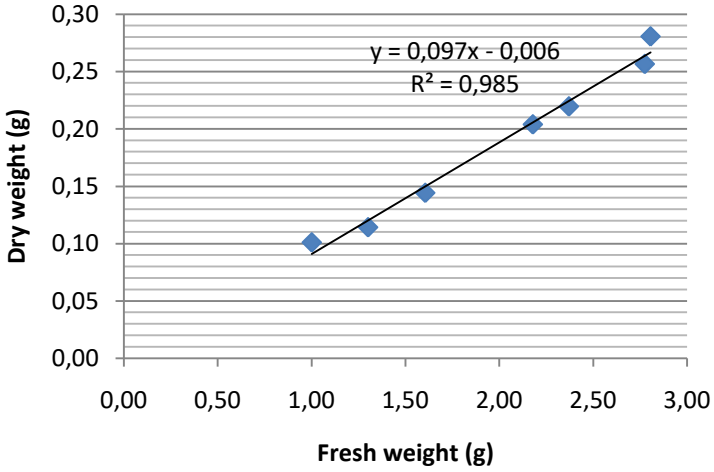


Figure 4. 13 *S. corrugata* hairy roots linear correlation: Increase of fresh weight in relation to the increase of dry weight during 6 weeks in batch culture.

Figure 4. 14 Hairy roots 6 week old, grown in liquid MS0 medium showing the dark brown central net non viable and the external emerging roots viable.

Conductivity of the tissue culture medium corresponded to the concentration of total components. On It is possible to observe the decrease of the conductivity from the beginning (T0) 5.60 mS/cm to the 5th week to 4.95 mS/cm and accompanied by an increase during the last week (5th to 6th week) from 4.95 to 5.24 mS/cm (**Fig. 4.15**). This gradual decrease of medium conductivity during the experiment can be attributed mainly to the consumption of nutrients and production of secondary metabolites. The (**Fig. 4.16**) showed the linear negative correlation between the medium conductivity and *S. corrugata* hairy root fresh weight. The increase of conductivity during the last week (5th to 6th) could be caused by a cell lyses due to senescence or death, hence the presence of turbid a light brown aspect of the medium.

During the growth of *S. corrugata* hairy root, the pH evolution of the medium presented a triphasic curve; the first phase form T0 to the 3rd week, the pH decrease from 5.12 to 4.02 with a severe decrease during the first week; the second phase from the 3rd to the 5th week the pH increase to reach 4.56, and became almost stable during the last week (**Fig. 4.15**).

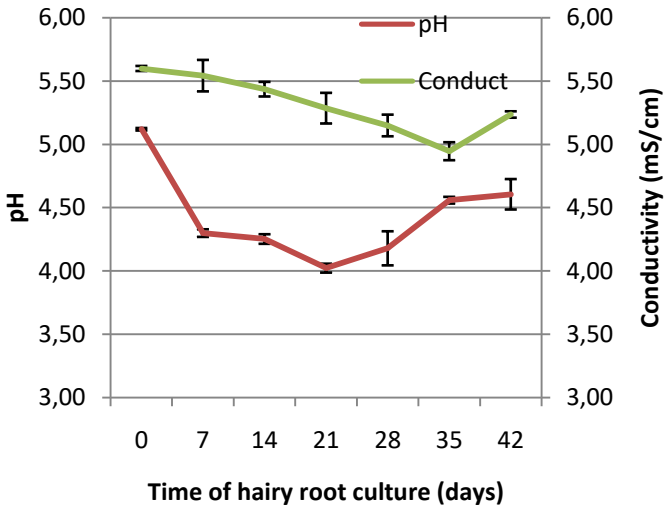


Figure 4. 15 Evolution of medium conductivity and pH at different stages of growth. Determinations were recorded every 7 days.

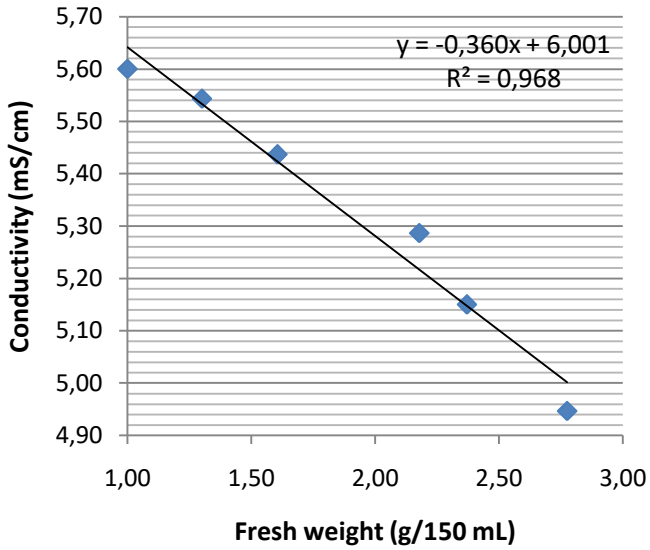


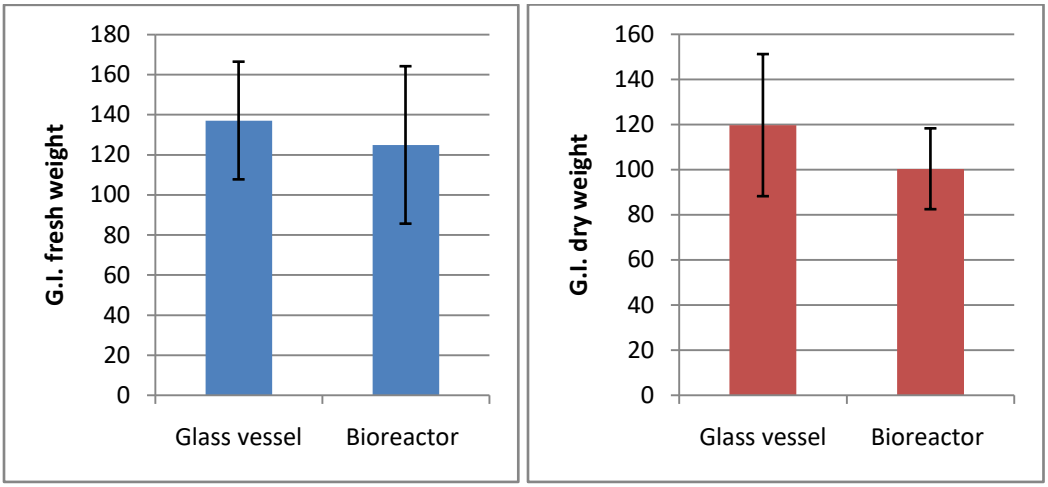
Figure 4. 16 Decrease of medium conductivity in relation to the increase of hairy root fresh weight during the 5 first weeks of culture.

4.1.1.2.6. Hairy root growth into the bioreactor RITA®

4.1.1.2.6.1. Container evaluation for biomass production

The comparison of growth performance of hairy roots in glass vessel or bioreactor is a huge step to be evaluated before the scale up production of biomass. The *S. corrugata* hairy root

biomass production during 28 days in glass vessel and bioreactor (RITA) did not show a significant difference between fresh and dry weights (**Fig. 4.17**). Both have a fresh and dry growth index of 120 to 140 and 100 to 120 in 28 days respectively.



(A) (B)

Figure 4. 17 Growth index of the biomass of *S. corrugata* hairy root into glass vessel and bioreactor (A) Fresh weight (B) Dry weight. Values represent the mean ± standard deviation (SD) n=3.

4.1.1.2.6.2. Effect of different medium formulations on hairy root growth

The growth rate of *S. corrugata* hairy root culture in different media (MS0, B5, WPM or in their half strength ½ MS, ½ B5 and ½ WPM) was measured after 30 days. As shown in **Fig. 4.18** A, among the six basal media tested, ½ WPM medium supported the best growth of fresh material 7.3 ± 0.3 g/150 mL that means (47.3 ± 2 g/L). Although, dry biomass production in ½ WPM medium was higher than MS0 medium, this difference was not statistically significant (**Fig. 4.18** B). The fresh biomass produced in the presence of full strength (MS0, B5 and WPM) and ½ B5 medium, did not show any significant difference. The ½ MS0 showed the lowest result on the fresh and dry biomass growth of *S. corrugata* with just 5.1 ± 0.6 g/150 mL (34 ± 4 g/L) and 0.37 ± 0.01 g/150 mL (2.46 ± 0.8 g/L) respectively. The B5 and half strength B5 (½ B5) media did not show any differences in biomass production, the means of fresh weight were 6.0 ± 0.4 and 6.0 ± 0.4 g/150 mL, and of dry weight were 0.43 ± 0.05 and 0.44 ± 0.06 g/150 mL respectively.

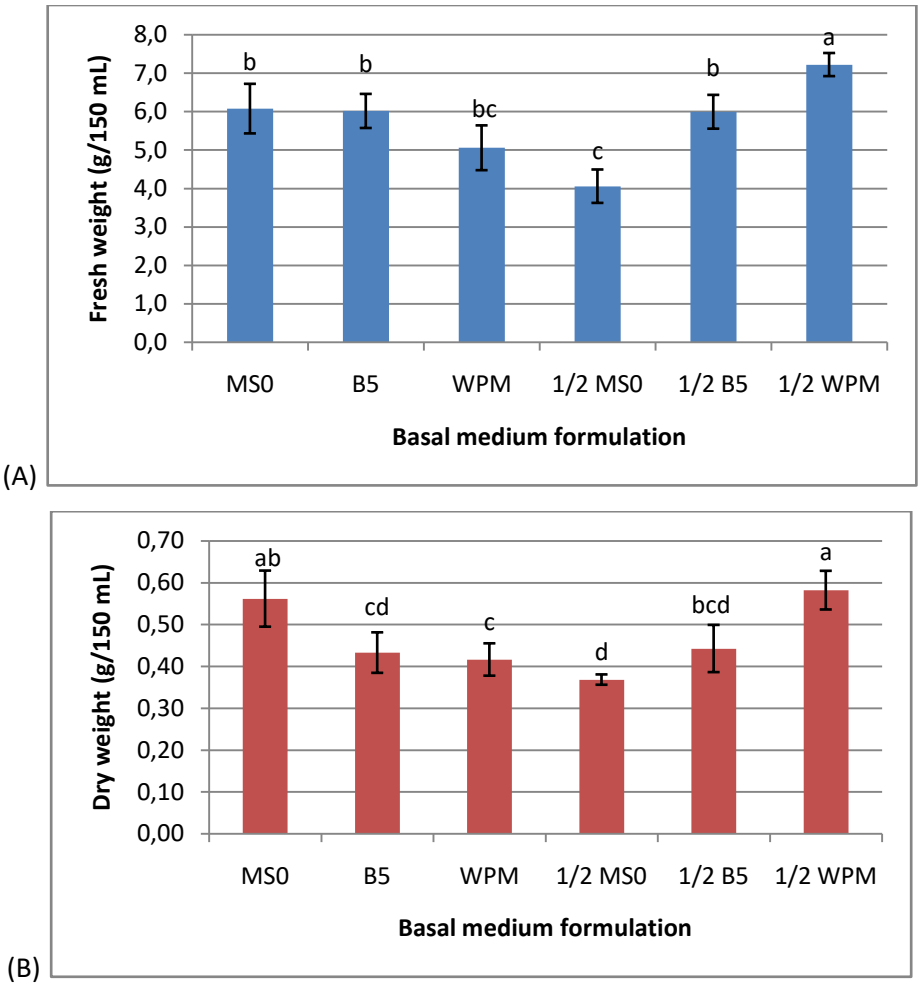
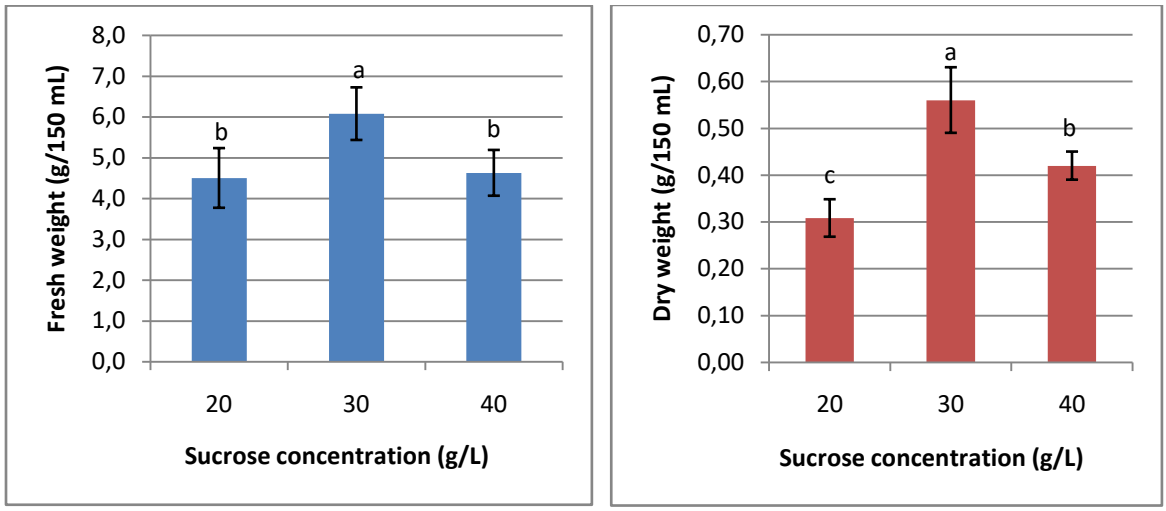


Figure 4. 18 Effect of different medium formulations on hairy root growth. (A) Fresh weight; (B) dry weight. Values represent the mean ± standard deviation (SD), n = 4; different letters identify values which differ at *p ≤ 0.05*.

4.1.1.2.6.3. Effect of initial sucrose concentration on hairy root growth

In general, sucrose is the carbon source necessary for *in vitro* tissue culture. The initial sucrose concentrations tested were 20, 30 and 40 g/L in MS0 using TIS RITA® bioreactor. The results for biomass production after 30 days of culture are shown in (**Fig. 4.19**). Sucrose concentration had a significant influence on the growth of transformed roots; growth was the fastest in medium containing 30 g/L sucrose reaching 6.1 ± 0.6 g/150 mL means 40.67 ± 4 g/L of fresh weight. The dry weight was about 180 % and 133% more than those from media containing 2% and 4% of sucrose respectively. The growth was limited at 20 and 40 g/L sucrose. No significant difference was observed between fresh weight growth with 20 or 40 g of sucrose. The evaluation of dry

weight showed a significant difference among all the treatments with value of 0.56 ± 0.07 g/150 mL and 0.31 ± 0.04 g/150 mL respectively at 30 and 20 g/L of sucrose.



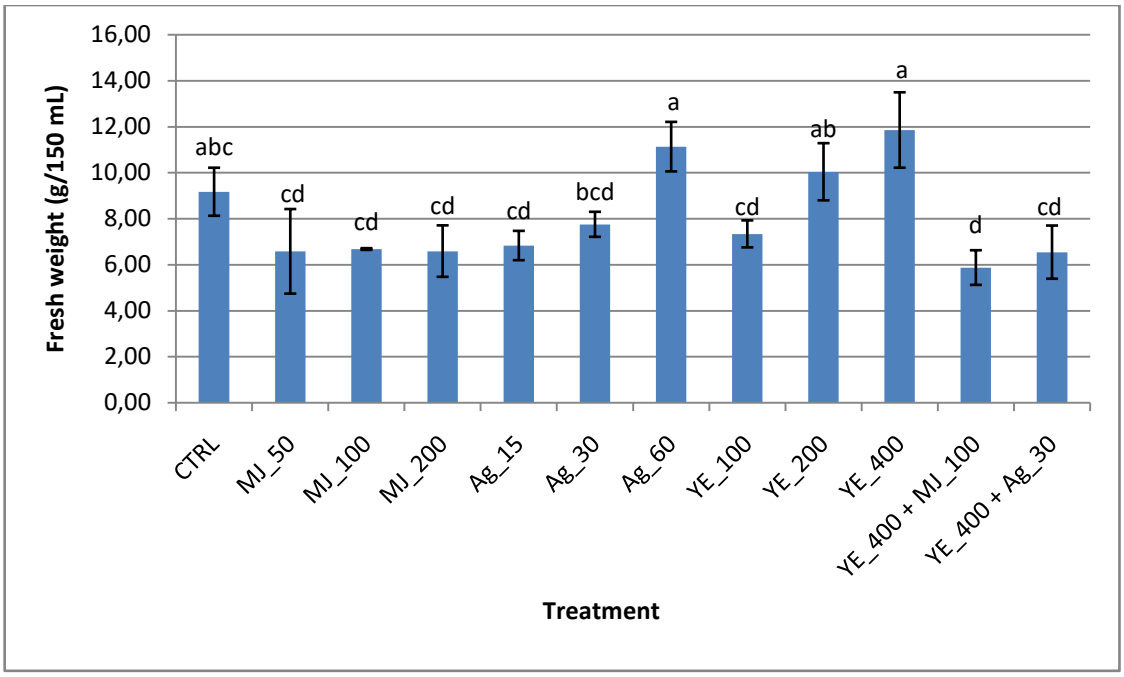
(A) (B)

Figure 4. 19 Effect of sucrose concentration on hairy root growth (A) fresh weight; (B) dry weight. Values represent the mean ± standard deviation (SD), n = 4; different letters identify values which differ at *p ≤ 0.05*.

4.1.1.2.6.4. Elicitation of Hairy roots with different elicitors

As seen in **Fig. 4.20**, the elicitor treatment enhanced the hairy root biomass growth along with the increase of elicitor concentrations: from 6.83 ± 0.64 to 11.13 ± 1.08 g/150 mL of FW or from 0.52 ± 0.05 to 0.79 ± 0.08 g/150 mL of DW with 15 mg/L and 60 mg/L with Ag, and from 7.34± 0.59 to 11.85 ± 1.64 g/150 mL or from 0.59 ± 0.04 to 0.81 ± 0.05 g/150 mL of DW with 100 mg/L and 400 mg/L with YE respectively. No significant differences were achieved with MJ elicitation. The combination of YE 400 mg/L treatment with MJ and Ag at the concentration 100 µM and 30 µM respectively decreased the biomass growth than that of the untreated control culture. In the **table 4.11**, it was reported the values of medium pH and conductivity. It has also been noted a decrease in conductivity with the increase of biomass in the case of root treated with YE and Ag as reported in **Fig. 4.21**.

(A)



(B)

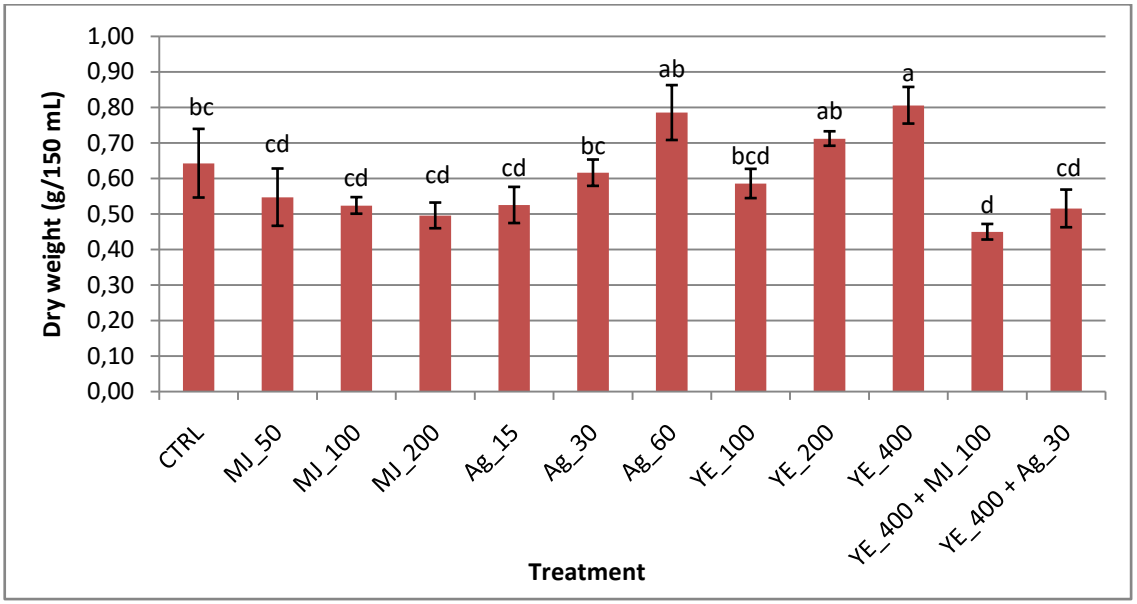


Figure 4. 20 Effects of different concentration of elicitors on *S. corrugata* hairy root biomass production: (A) Fresh weight, (B) Dry weight. Values represent the mean ± standard deviation (SD) n=3; different letters identify values which differ at $p \leq 0.05$.

Table 4. 11 Medium pH and conductivity values of *S. corrugata* hairy root growth with the concentration of elicitor.

Treatments	pH	Conductivity (µS/cm)
CTRL	3.94 ± 0.14	759 ± 203
MJ_50	4.63 ± 0.21	1074 ± 199
MJ_100	4.86 ± 0.39	1086 ± 80
MJ_200	4.31 ± 0.93	1250 ± 314
Ag_15	4.23 ± 0.09	1089 ± 98
Ag_30	4.48 ± 0.12	1012 ± 108
Ag_60	4.49 ± 0.36	830 ± 42
YE_100	4.10 ± 0.09	847 ± 108
YE_200	4.20 ± 0.15	755 ± 55
YE_400	3.87 ± 0.21	672 ± 5
YE_400 + MJ_100	4.37 ± 0.07	1147 ± 88
YE_400 + Ag_30	4.25 ± 0.34	1167 ± 109

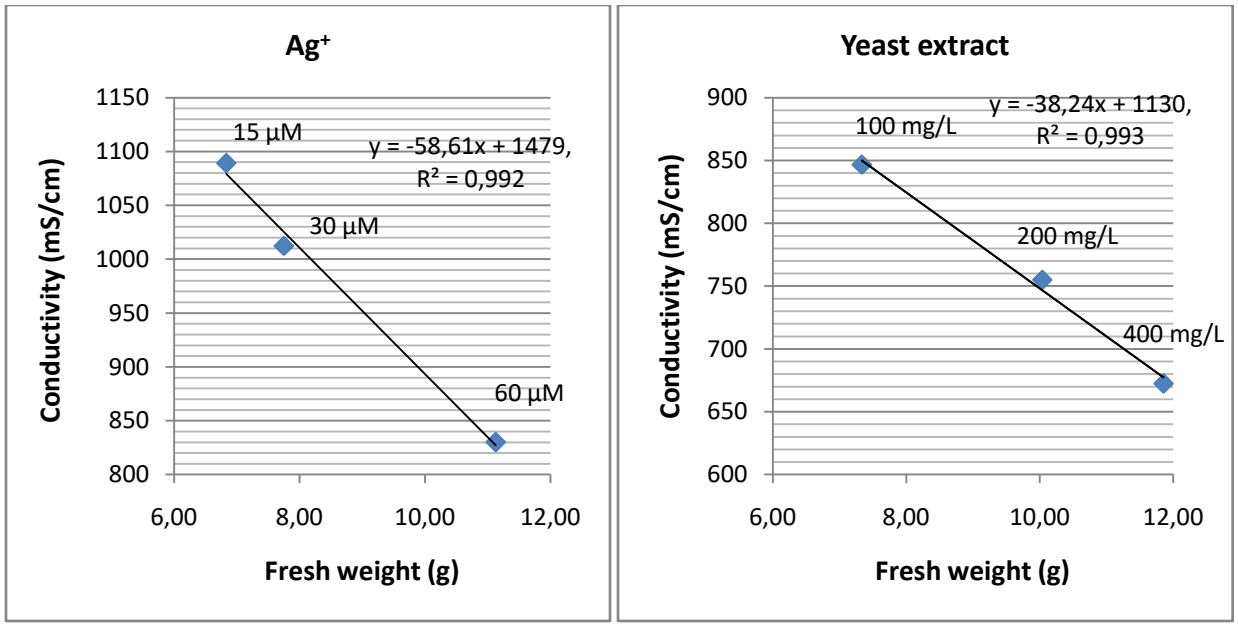


Figure 4. 21 Relationship between the medium conductivity and hairy root fresh weight elicited with different concentration of elicitors.

4.1.1.2.6.5. Scale-up production of hairy roots biomass

Although the biomass production was similar in bioreactor or glass vessel, it was used for the production of large biomass. Owing to the possibility to authomize the culture sequence. After the identification of the best growth parameters, growth of total biomass for secondary metabolite extraction was done following a process showing in **Fig. 4.22**. Once the fresh weight into the glass vessel reached about 5 g, the material was transferred into the bioreactor containing the MS0 medium 150 mL After three months cultivation, changing the medium every 10 to 15 days with fresh one, the hairy roots showed non homogeneous distribution of the tissues; the external part of the hairy root net was alive and white/yellowish, while the central part was dark showing a dense net clump of senescent root tissue that caused a block of liquid medium flow and thus limitation of oxygen availability (**Fig. 4.23**). The culture medium began to turn brown, a sign of aging of the hairy roots. At this moment, the hairy roots were harvested, weighted and dried by lyophilization. All the different clones SCO-HR-FA8, SCO-HR-FA13 and SCO-HR-FL7 were tested for this production and the results of biomass production are showing in **Table 4.12**.

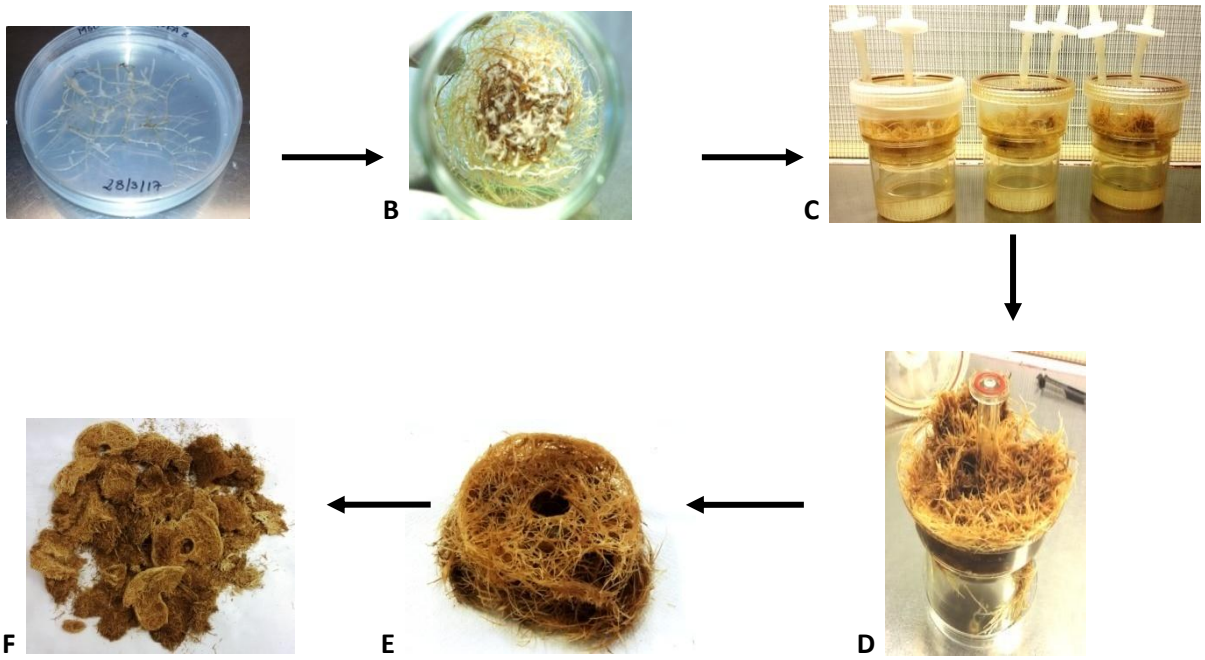


Figure 4. 22 Biomass production sequences of *S. corrugata* hairy roots in MS0 medium. A = Culture in Petri-dish onto solid medium. B = Liquid culture in vessel. C = Culture in bioreactor: RITA® bioreactors. D =Sampling. E =Fresh product F = Dry product.

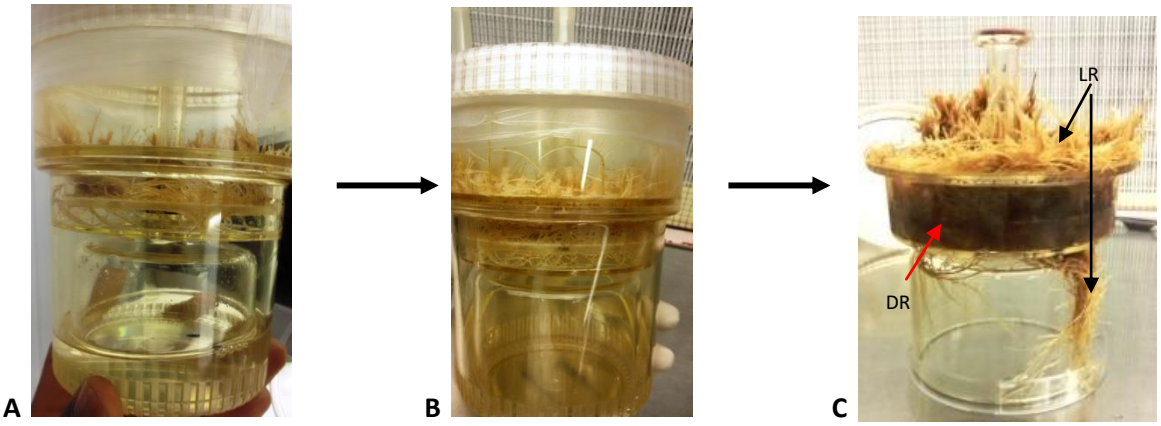


Figure 4. 22 Evolution of aspect of hairy root into the bioreactor. A: after 2 weeks; B: after 6 weeks, C: after 3 months (LR) living roots (DR) dead roots.

Table 4. 12 Biomass production of different clones of *S. corrugata* hairy root in RITA® bioreactors

Clones	Number of bioreactors	Initial FW in 150 ml (values in 1L)	Final FW in 150 mL (g)	Final FW in 1 L (g)	Total FW produced (g)	Dry Weight DW (g)
SCO-HR-FA8	13	5 (33)	37,5	250	487.8	45.98
SCO-HR-FA13	4		35.9	239	143.4	16.53
SCO-HR-FL7	1		23.3	155	23.3	2.40

4.1.1.3. Phytochemical analysis of hairy roots

4.1.1.3.1. Extracts preparation

The dried *S. corrugata* hairy roots of all selected clones were reduced to powder and extracted with methanol and the residue was evaporated under reduced pressure. The extracts were transferred to the glass and dried at room temperature in the laboratory. The extraction of different clone was showed in to the **table 4.13**. The extractive yield between the clones SCO-HR-FA8 and SCO-HR-FA13 derived from ATCC 15835 was almost 36.5 and 36.1 respectively and superior to 32.5 came from LBA 9402 (SCO-HR-FL7).

Table 4. 13 Methanolic extraction of *S. corrugata* hairy root clones

***A. rhizogenes* strains**	**Clones**	**Dry weight (g)**	**Methanolic extract (g)**	**Extraction yield (%)**
ATCC 15834	SCO-HR-FA8	45.98	16.8	36.5
	SCO-HR-FA13	16.53	5.97	36.1
LBA 9402	SCO-HR-FL7	2.40	0.78	32.5

4.1.1.3.2. Fruticuline A and demethylfruticuline A analysis on *Salvia corrugata* hairy roots.

The qualitative analysis of the methanolic extract of the three clones of *S. corrugata* hairy root with TLC did not showed a relative band detection of fruticuline A and demethylfruticuline A represented on the chromatography plate of *S. corrugata* shoot extract by the green color band (**Fig. 4.24)**. However, analyzing the TLC, there was not obvious variation in the chromatographic profiles between the three clones extracts. It has also been found, the presence of many bands, evidence of a rich variety content of secondary metabolites.

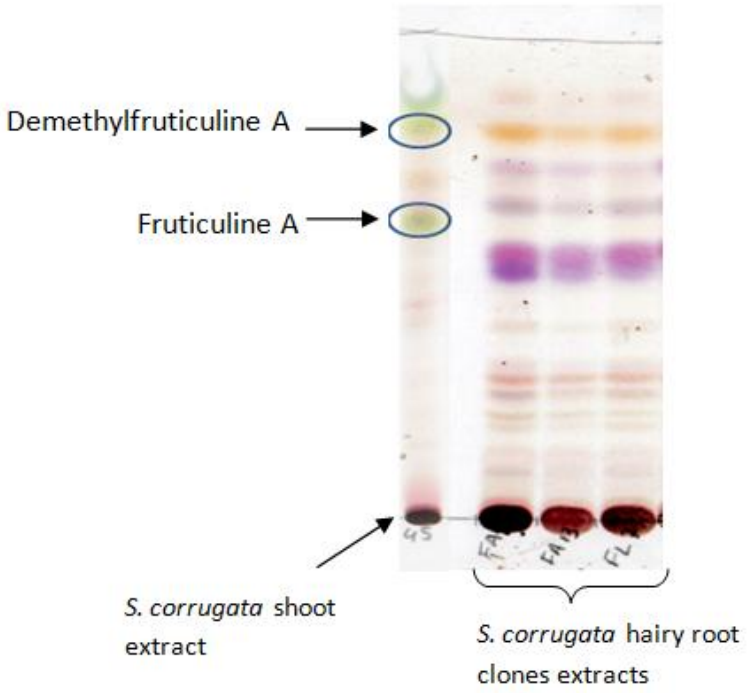


Figure 4. 23 TLC comparison of different clones of *S. corrugata* hairy root and shoot extract of showing the **demethylfruticuline A and fruticuline A.**

The quantitative analysis on LC-MS/MS of demethylfruticuline A and fruticuline A retention time 2.97 min and 3.63 min respectively, conducted on all *S. corrugata* hairy root clone extract (at the concentration of 15 mg/mL) showed a peak intensity of clone SCO-HR-FA8 similar to the blank solution (**Fig 4.25**), indicating a non production of this compounds into transformed *S. corrugata* roots confirmed previously by the TLC analysis (**Fig. 4.24**). Representative chromatograms of the LC-MS/MS (MRM) profile of fruticuline A and demthylfruticuline A are reported in **Annex 5**.

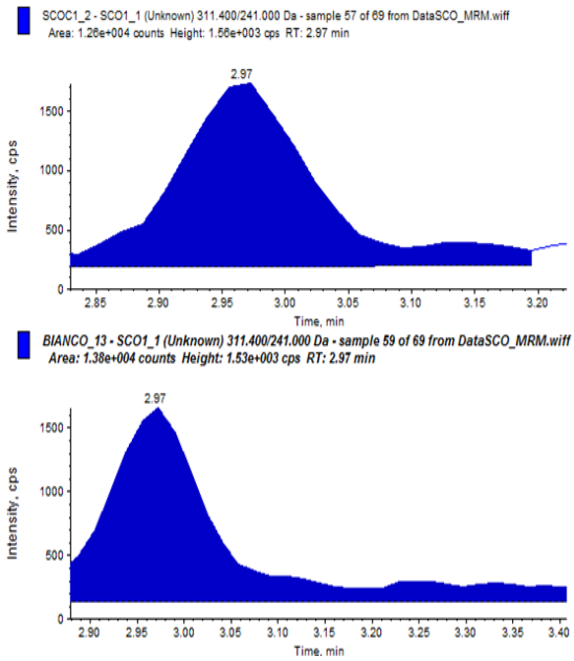


Figure 4. 24 Comparison of peaks intensity of demethylfruticuline A into the *S. corrugata* hairy root clone SCO-HR-FA8 and into the blank solution.

The TLC analysis of methanolic extract of the clones SCO-HR-FA8 of *S. corrugata* hairy root elicitated with different types of elicitors and at all the concentration used (see **Table 3.5**) did not showed a relative band detection of fruticuline A and demethylfruticuline A represented on the chromatography plate of *S. corrugata* shoot extract by the green color band (**Fig. 4.26**). On the TLC, showed a similar range composition of compounds for each extract from *S. corrugata* hairy root elicitated with different elicitors.

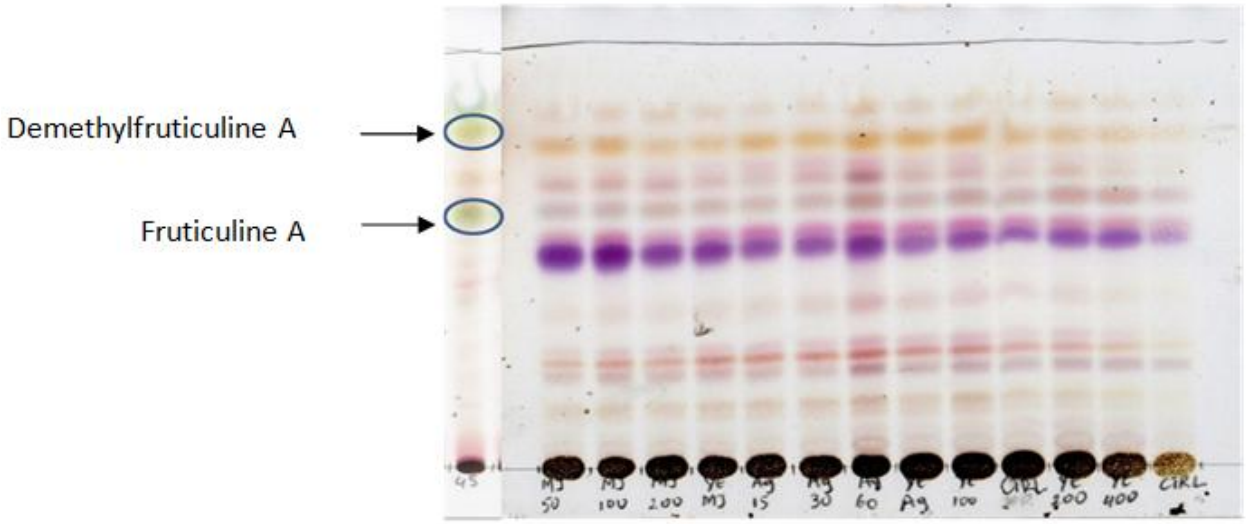


Figure 4. 25 TLC comparisons of different extracts of *S. corrugata* hairy root elicitated with different type and concentration of elicitor, and shoot extract showing the demethylfruticuline A and fruticuline A.

4.1.1.3.3. Fractionation of *Salvia. corrugata* hairy roots

The methanolic extract of the SCO-HR-FA8 biomass (16.8 g) was fractionated by Si gel MPLC (Medium Pressure Liquid Chromatography) to obtain 16 semi-purified fractions. The final TLC chromatograms from *S. corrugata* hairy roots revealed several chemical compositions (**Fig. 4.27**). These fractions were dried at room temperature and allowed to obtain a dry mass represented in the **Table 4.14**.

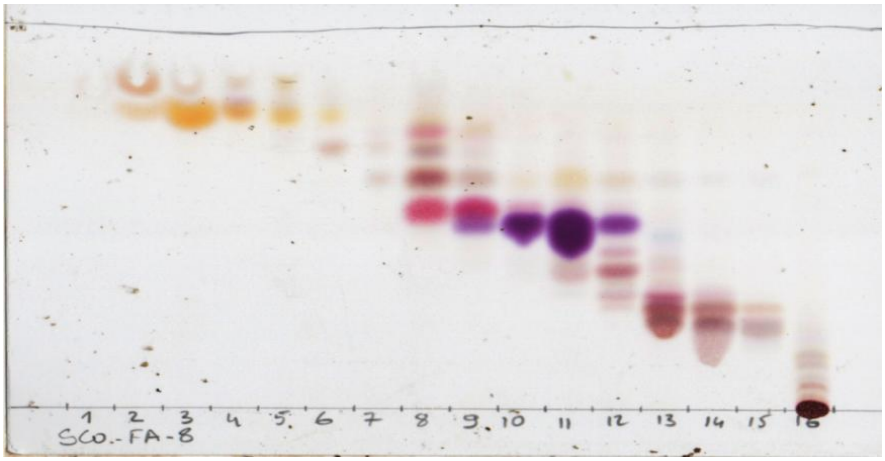


Figure 4. 26 TLC chromatogram of chemical composition of different fractions of *S. corrugata* hairy root clone SCO-HR-FA8.

Table 4. 14 Separation of methanolic extract of *S. corrugata* hairy root clone SCO-HR-FA8.

Fraction	Solvents	Initial fraction	Final fraction	Weight (mg)
1	esano	1	13	31
2	CHCl3	14	14	64.7
3	CHCl3	15	16	107.6
4	CHCl3	17	18	29.3
5	CHCl3	19	22	50.6
6	CHCl3	23	24	40.6
7	CHCl3	25	25	19.1
8	CHCl3	26	29	267.5
9	CHCl3	30	34	50.2
10	CHCl3/MeOH 95:5	35	40	69.5
11	CHCl3/MeOH 95:5	41	42	218.5
12	CHCl3/MeOH 95:5	43	45	164.2
13	CHCl3/MeOH 95:5	46	47	239.9
14	CHCl3/MeOH 95:5	48	51	253.9
15	CHCl3/MeOH 95:5	52	54	71.5
16	MeOH	55	57	9585.6

Fraction VI (eluted with $CHCl_3$, from 0.78 to 0.96 L) was purified by semi-preparative RP HPLC (eluents A: H_2O, B: CH_3OH, gradient: B 5% at time 0 min, B 100% at time 61 min, B 100% at time 70 min) to obtain horminone (1.5mg) and 7-acetyl-horminone (1.7mg). Ferruginol and the new 19[4→3]abeo-O-demethyl-14-hydroxy-cryptojaponol were also isolated.

Ferruginol

19[4→3]abeo-O-demethyl-14-hydroxy-cryptojaponol

4.1.1.4. Antibacterial activities

The antibacterial activity of the hairy root methanolic extract of *S. corrugata* and sixteen semi-purified fractions obtained by MPLC was assessed by determining Minimal Inhibitory Concentration (MIC) values on a total of twenty two clinical isolates. The results of susceptibility testing against the strains of *Staphylococci* and *Enterococci* studied are given in **Table 4.15** and **Table 4.16**, respectively. Methanolic extract and the following twelve semi-purified fractions 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 16 were surprisingly inactive (MIC values > 128 µg/mL) when assayed against all the tested strains, while the fraction 11, although not active against *Staphylococci*, was endowed with different potency on all *Enterococci* strains analyzed here, with MICs values from 16 to 32 µg/mL. However, three semi-purified fractions, 13, 14 and 15, displayed a very huge distribution of MIC values, ranging from 8 µg/mL to 64 µg/mL against various strains of *Staphylococci*. The MIC values 8 µg/mL was observed against *S. aureus, S. epidermidis, S. lugdunensis, S. warneri* (oxa-R) and *S. stimulans* (oxa-R) when treated with fraction 14. This MIC value of 8 µg/mL was also found against *S. lugdunensis, S. warneri* (oxa-R) when treated with fractions 13 and 14 respectively. Interestingly, these semi-purified fractions 13, 14 and 15 displayed a MIC value ranging from 4 µg/mL to 32 µg/mL against Enterococci. The MIC values 4 µg/mL was observed against *E. faecalis, E. faecium* and *E. casseliflavus* (VRE) treated with fraction 14 and against the strain *E. gallinarum* (VRE) treated with the fraction 13. This suggested a relatively more potent action of these four semi-purified fractions against *Enterococci* than *Staphylococci*. *E. coli* was the most resistant strain against methanolic extract and all semi-purified fractions with all MIC values obtained greater than 128 µg/mL.

The new compound 19[4-3]abeo-O-demethyl-14-hydroxy-cryptojaponol showed a very wide range of activity against *Enterococci* (MIC values 1 – 128 µg/mL) as reported in **table 4.17**. This compound displayed a MIC value of 1 µg/mL against *E. durans* and 4 µg/mL against *E. Faecium* and *E. casseliflavus* vancomycin resistant strain. The MIC value of 128 µg/mL was observed against *E. faecalis* strains.

Table 4. 15 Minimal inhibitory concentrations (MICs) of *S. corrugata* hairy root methanolic extract and different fractions against *Staphylococci*. MIC values are expressed in µg/mL.

Strains	HR MeOH extract	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 16	11	13	14	15
***S. aureus* 18 (oxa-R)**	> 128	> 128	> 128	32	16	32
***S. aureus* 6**	> 128	> 128	> 128	64	8	16
***S. aureus* 91**	> 128	> 128	> 128	32	8	32
***S. epidermidis* 22 (oxa-R)**	> 128	> 128	> 128	16	16	32
***S. epidermidis* 178 (oxa-R and lnz-R)**	> 128	> 128	> 128	32	16	16
***S. epidermidis* 93**	> 128	> 128	> 128	32	8	32
***S. saprophyticus* 41**	nt	nt	nt	64	16	16
***S. lugdunensis* 129**	nt	nt	nt	16	8	8
***S. warneri* 137 (oxa-R)**	nt	nt	nt	8	8	32
***S. simulans* 163 (oxa-R)**	nt	nt	nt	16	8	64
***S. capitis* 192 (oxa-R)**	nt	nt	nt	32	16	64
***S. haemolyticus* 193 (oxa-R)**	nt	nt	nt	64	16	32
***S. hominis* 194 (oxa-R)**	nt	nt	nt	32	16	16

nt: not tested.

oxa-R: Oxacillin resistance

lnz-R: Linezolid resistance

Table 4. 16 Minimal inhibitory concentrations (MICs) of *S. corrugata* hairy root methanolic extract and different fractions against *Enterococci* and *E. coli*. MIC values are expressed in µg/mL.

Strains	**HR MeOH extract**	**1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12 and 16**	**11**	**13**	**14**	**15**
***E. faecalis* 1 (VRE)**	> 128	> 128	32	16	8	16
***E. faecalis* 92**	> 128	> 128	16	16	**4**	16
***E. faecium* 2**	> 128	> 128	16	8	**4**	8
***E. faecium* 152 (VRE)**	> 128	> 128	32	16	8	16
***E. gallinarum* 105 (VRE)**	nt	nt	32	**4**	16	16
***E. durans* 113**	nt	nt	16	8	8	8
***E. avium* 122**	nt	nt	32	16	16	16
***E. casseliflavus* 184 (VRE)**	nt	nt	16	32	**4**	8
E. coli	> 128	> 128	> 128	> 128	> 128	> 128

nt: not tested

VRE: Vancomycin resistance

Table 4. 17 Minimal inhibitory concentrations (MICs) of 19[4→3]abeo-O-demethyl-14-hydroxy-cryptojaponol against *Enterococci*. MIC values are expressed in µg/mL.

Strain	New compound
E. faecalis MB1**	**128**
E. faecalis MB92	**>128**
E. faecium MB2	**4**
E. faecium MB152**	**8**
E. durans MB113	**1**
E. gallinarum MB105**	**32**
E. avium MB119	**16**
E. casseliflavus MB**	**4**

** Vancomycin resistant strain.

Based on the minimal values of MICs found on to the four semi-purified fractions 11, 13, 14 and 15, their ulterior purification could show potential antibacterial compounds.

4.1.2. Results of *Salvia tingitana* Etl.

4.1.2.1. *Salvia tingitana* shoot establishment

4.1.2.1.1. In vivo root induction

After 40 days of culture in greenhouse, the explants immersed by in a solution of K-IBA 3000 ppm developed complete root apparatus (**Fig. 4.28**). Complete plants were then transferred in pot (diameter 14 cm) in order to produce fresh root biomass for metabolite extraction.

A  B 

Figure 4. 27 *In vivo* rooting of *S. tingitana* cuttings. (A) shoot explants (B) new plant with roots.

4.1.2.1.2. Sterilization and in vitro shoot induction

The explants used for the *in vitro* culture establishment were apical portions or internodes with at least one node. In **Table 4.18**, the sterilization efficiency expressed as the explants' viability after 4 weeks of culture. As we can see, the pre-treatment with tap water for 15' combined to the sterilization with the concentration of NaClO 1% or 2% for 15' were able to guarantee a proper sterilization and maintain the viability of shoots (sequences 5 and 6). The pre-treatment with ethanol 70% for 1 minute completely reduced the viability of explants (sequences 1 and 2); the concentrations of NaClO less than 0.5% for 15' and 1% during 10' were not enough to obtain clean and sterilized explants. Micropropagated shoots rooted easily when transferred on MS medium without BA (**Fig. 4.29**).

Table 4. 18 Contamination and viability percentage, of micropropagated *S. tingitana* cultured on MS medium supplemented with 1.33 µM of BA evaluated after 4 weeks depending on the sterilization sequence used.

Sequence	Pre-treatment and sterilization conditions	% contamination	% viability of shoot
1	EtOH 70% 1'; NaClO 0.5% + tween 20 1% for 10'	25	0.0
2	EtOH 70% 1'; NaClO 1% + tween 20 1% for 10'	50	0.0
3	soapy water 10'; NaClO 0.5% + tween 20 1% for 15'	75	100.0
4	Tap water 15'; NaClO 0.5% + tween 20 1% for 15'	33.3	100.0
5	**Tap water 15'; NaClO 1% + tween 20 1% for 15'**	**0**	**100.0**
6	**Tap water 15'; NaClO 2% + tween 20 1% for 15'**	**0**	**100.0**

A  **B**  **C** 

Figure 4. 28 *In vitro* shoot of *S. tingitana* cultured in agarized medium. A: sterilized shoot, B: micropropagation, C: shoot showing roots.

4.1.2.1.3. In vitro seed germination

After two weeks, the first group pre-treated with 70% of ethanol did not showed germination. However, the second group sterilized only with 0.2 % (v/v) sodium hypochlorite solution (NaClO) showed 40% of germination. Geminated seeds were transferred in glass vessel containing the same medium and at the following environmental conditions: 23 ± 2 °C, 16 h lighting photoperiod at 30 µE $m^{-2}s^{-1}$ (**Fig. 4.30)**.

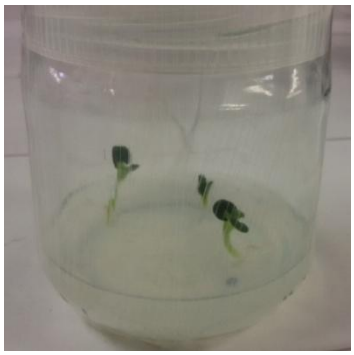
(A)  (B) 

Figure 4. 29 *S. tingitana* seed germination. (A): at 10 days of germination in MS hormone-free medium in dark. (B) day 15 seedlings transferred on MS supplemented with 1.33 µM of BA in light.

4.1.2.1.4. In vitro root induction

As reported in **table 4.19**, shoots culture after 30 days shoots were best rooted on MS medium supplemented with IBA (1.33 µM) which induced a maximum of 1.17 roots per shoot with 30.67 mm root length in 66.7% of shoot. Shoots cultured on MS with. IAA (1.33 µM) developed root as compared to MS0. However, 83.33% of shoots developed callus at the cutting base inserted into the culture medium as shown in **Fig. 4.31**. Shoots developed 0.71 roots per shoot in auxin-free MS medium (control) and showed stunted growth (25 mm root length). However, any callus formation was observed on shoots cultured in MS0 or MS supplemented with IBA.

Table 4. 19 Effect of different auxin on *in vitro* rooting of *S. tingitana* shoots after 30 days of culture. Values represent the mean of 6 replicates ± standard error (SE).

Treatment	weight (g)	shoot length (mm)	Rooting %	N. Root	root length (mm)	callus % of callus formation
MS0	0.15 ± 0.02	24.86 ± 1.11	50	0.71 ± 0.33	25.00 ± 6.47	0
IBA (1.33µM)	0.20 ± 0.04	30.67 ± 3.62	66.7	1.17 ± 0.48	40.43 ± 5.59	0
IAA (1.33µM)	0.28 ± 0.04	36.83 ± 3.08	33.3	0.67 ± 0.42	16.17 ± 7.42	83.3

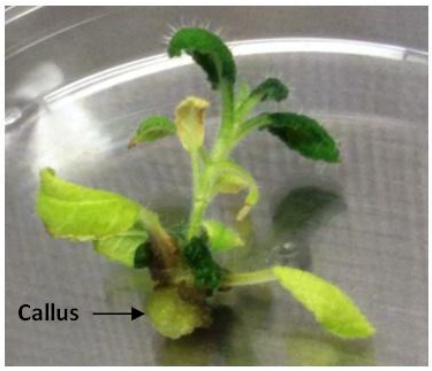


Figure 4. 30 Callus formation at the base of shoots grown in MS supplemented with IAA.

4.1.2.2. Establishment and analysis of callus from *Salvia tingitana* Etl

4.1.2.2.1. Sterilization and establishment of callus induction

Callus induction from leaf explants started between 2 weeks, the **table 4.20** reported the percentage of *S, tingitana* callus induction at different concentration of PGRs in dark and light condition after 4 weeks of culture. The highest frequency of callus induction (94.4%) was observed in dark condition in two media: the first supplemented with 0.5 mg/L of KIN and 0.5 mg/L of 2,4-D, and the second supplemented with 1 mg/L of KIN and 1 mg/L of 2,4-D. All media without 2,4-D did not showed any callus induction. The callus obtained was whitish color and hard (**Fig. 4.32**).

In light condition the highest frequency (61.1%) was observed with the combination of 1 mg/L of KIN 1 mg/L of 2,4-D. Like in dark, in the medium lacking of 2,4-D any callus formation was observed. Moreover, at the highest concentration of 2,4-D (5mg/L), no induction was also detected. The callus obtained presented mostly a green whitish color and hard and compact aspect (**Fig. 4.33**). In this study, it has been noticed that callus developed mainly at the level of the leaf central rib in the both photoperiod condition. Moreover, despite the presence of ascorbic acid, almost all the explants developed dark or brown necrotic areas around the explants base in light; yellow or greenish-yellow necrotic areas developed in dark condition, which in some cases led to explants senescence and death. However, if the browning did not extend over the entire tissue, it apparently did not inhibit callus induction.

After the transfer of a part of the callus on respective culture medium without 2,4-D on the same conditions, not somatic embryogenesis was developed. Based on percentage of induction and the behaviour quality of *S. tingitana,* the callus grown on medium supplemented with KIN and 2,4-D at

0.1:0.5 respectively with ascorbic acid and in dark was maintained for the further experiments. After several subcultures in this medium, the callus remained hard.

Table 4. 20 Callus induction from leaf of *S. tingitana* on MS medium supplemented with different levels and combinations of PGRs (KIN: 0, 0.1, 0.5, 1 and 2,4-D: 0, 0.5, 1 and 5 mg/L) in light and dark conditions. Data reported after 28 days. (*Quality of callus).

MS + Ascorbic acid 10 mg/L + PGRs (mg/L) indicated		**photoperiod in light**			
		16 h		**0**	
KIN	**2,4-D**	**% Induction**	**callus behaviour**	**% Induction**	**callus behaviour**
0	0	0	-	0	-
0	0.5	11.1	* Whitish Green	55.6	* Whitish
0	1	16.7	* Whitish Green	38.9	* Whitish
0	5	0	-	5.6	* Whitish
0.1	0	0	-	0	-
0.1	0.5	44.4	**Whitish Green	83.3	*** Whitish
0.1	1	16.7	**Whitish Green	77.8	** Whitish
0.1	5	0	-	33.3	*Whitish
0.5	0	0	-	0	-
0.5	0.5	22.2	** Brown	**94.4**	*** Whitish
0.5	1	27.8	** Brown	50	** Whitish
0.5	5	0	-	22.2	* Whitish
1	0	0	-	0	-
1	0.5	33.3	** Whitish Green	77.8	*** Whitish
1	1	**61.1**	*** Whitish Green	**94.4**	** Whitish
1	5	0	-	44.4	* Whitish

Among all factors investigated, the combination of the presence of 2,4-D and dark appear to best induce callus development.

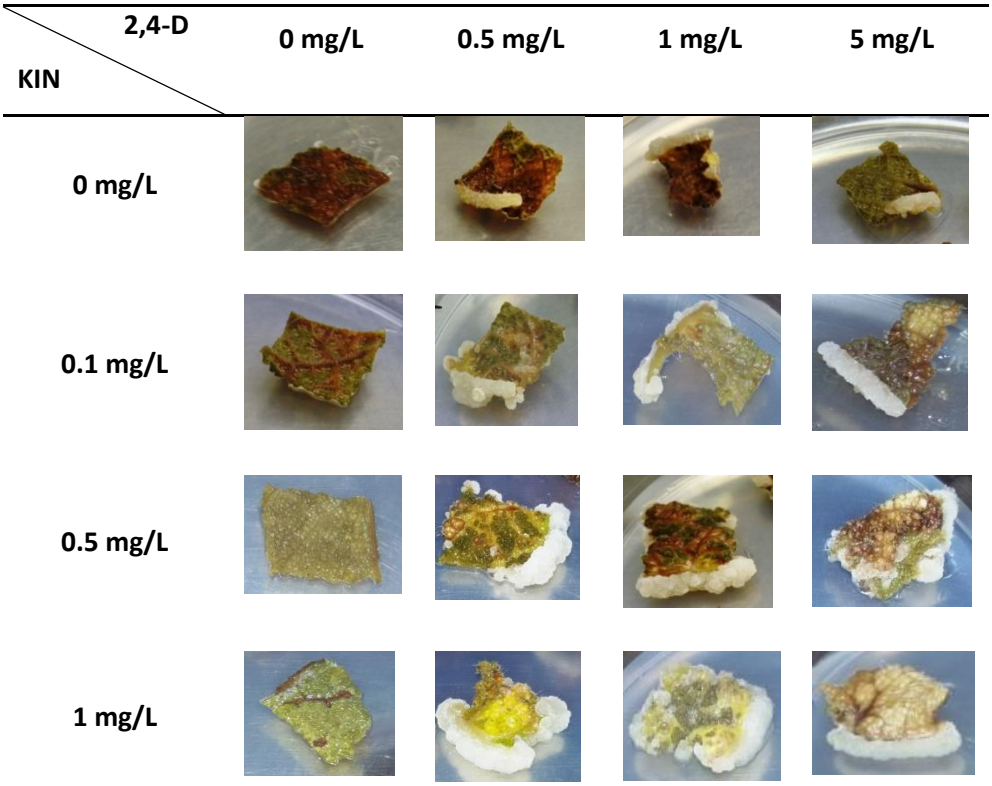


Figure 4. 31 *Salvia tingitana* callus developed from leaf explant in dark condition on MS supplemented with different combination of PGRs and 10 mg/l of ascorbic acid after 4 weeks.

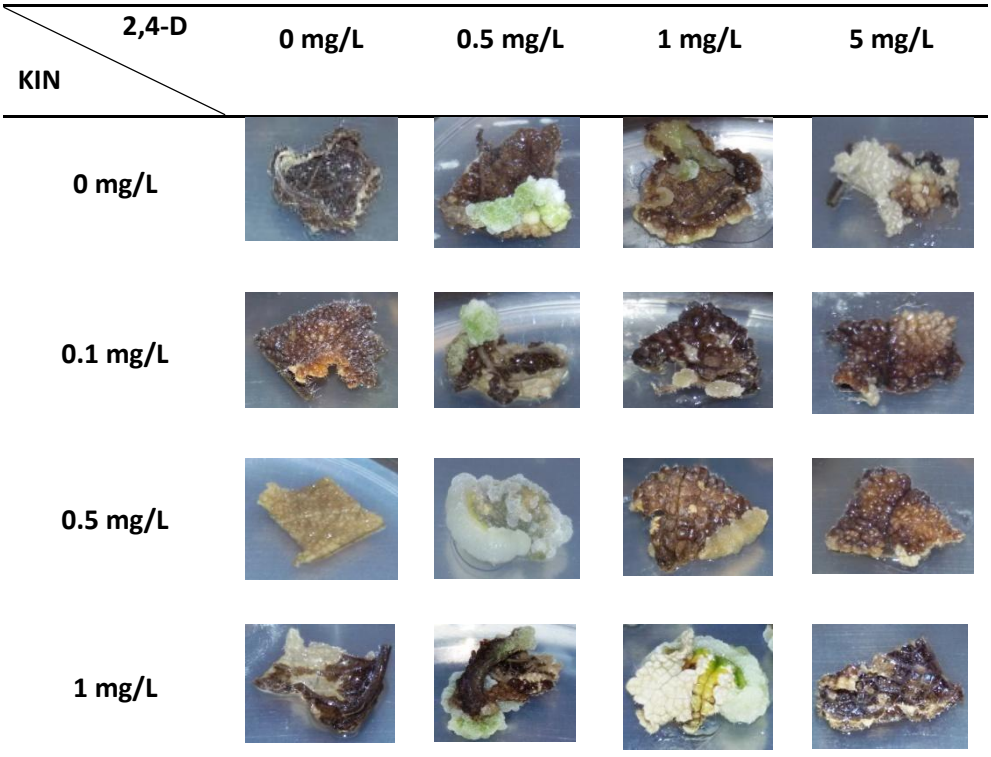


Figure 4. 32 *Salvia tingitana* callus developed from leaf explant in light condition on MS supplemented with different combination of PGRs and 10 mg/l of ascorbic acid after 4 weeks.

4.1.2.2.2. Callus viability

Intracellular esterases hydrolyze a fluorogenic substrate (fluorescein diacetate), that can pass through the cell membrane, where upon they cleave off the diacetate group producing the highly fluorescent product fluorescein. In our material, the FDA staining permitted to define the viability of calli developed from leaf tissues and the heterogeneous dimension of cell (**Fig. 4.34**). It is possible to observe alive (green color on fluorescent option) and dead (only on bright-field option) aggregate cell. It is important to make selection of the green material in order to maintained or discard the calli.

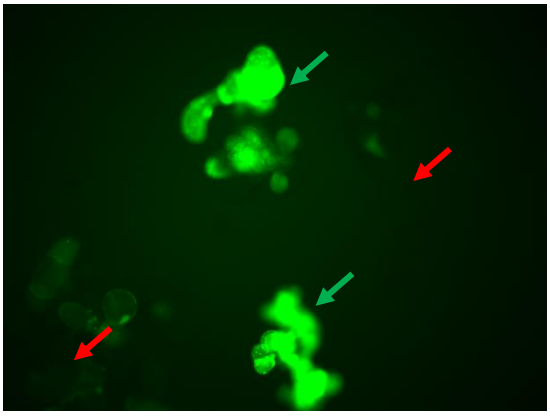
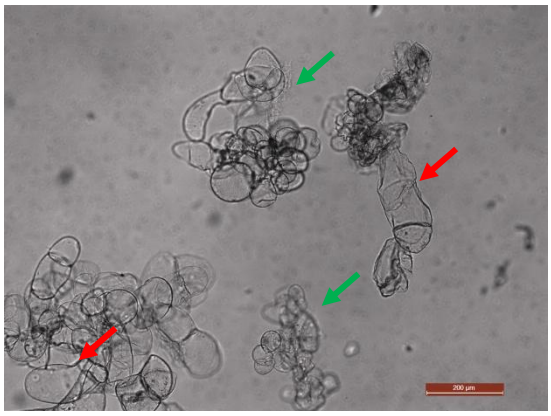
(A)  (B) 

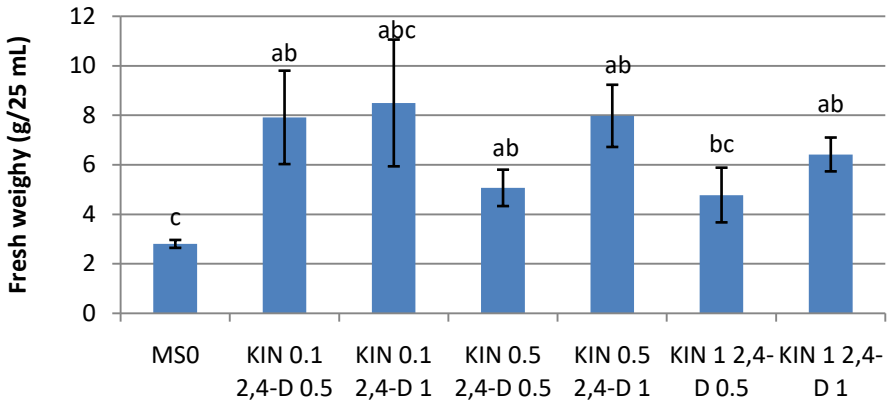
Figure 4. 33 FDA staining of *S. tingitana* callus. (A) fluorescent observation, (B) bright field observation with fluorescence microscopy (40×). Green arrows means living cell while red means dead cells.

It is important to make selection of the green material in order to maintained or discard the calli.

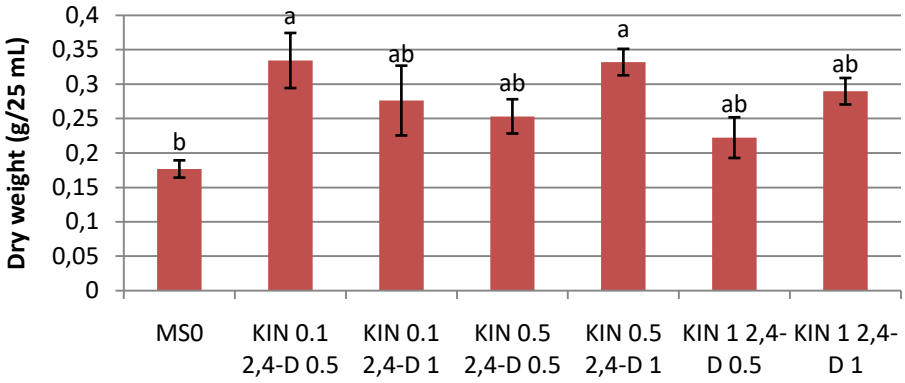
4.1.2.2.3. Influence of growth regulators on biomass production.

4.1.2.2.3.1. Effect of different concentration of KIN and 2,4-D.

In **Fig. 4.35,** the fresh weight and dry weight of callus were reported. It is possible to observe the worst performance in absence of PGRs and the good results in presence of KIN and 2,4-D at 0.1:0.5 and 0.5 :1 respectively. This result is statistically noted in the DW evaluation (**Fig 4.35.**B).



(A)



(B)

Figure 4. 34 Effect of different combination of plant growth regulators to *S. tingitana* callus biomass production. Data are reported as mean of six replicate ± SE, n=6. Different letters identify values which differ at *p ≤ 0.05*

From the result of the first experiment, the culture medium containing the combination of KIN 2.32 µM and 2,4-D 4.52 µM supplemented with 10 mg/mL of ascorbic acid was selected for the biomass production. It has been noted that after several subcultures in this selected medium, the calli have become friable as show in **Fig. 4.36**.

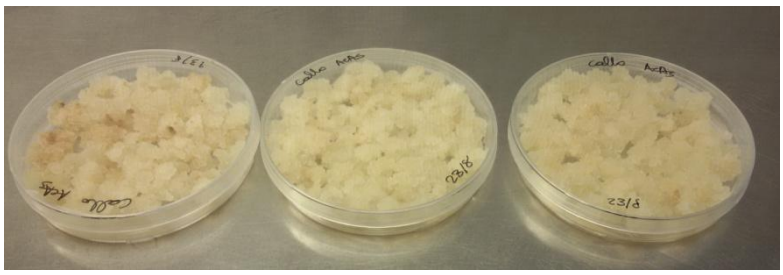


Figure 4. 35 Friable callus obtained after several subculture in MS medium supplemented with **KIN 2.32 µM and 2,4-D 4.52 µM supplemented with 10 mg/mL.**

4.1.2.2.3.2. Effect PGRs types

The **Fig.4.37** reported the fresh and dry weights of callus influence of KIN BA or MTP (2.32 µM); it is shown that the type of cytokinin did not influence statistically the *S. tingitana* callus fresh and dry biomass production after 35 day of culture. However, medium with KIN and MTP tended to favorite biomass growth in dark condition.

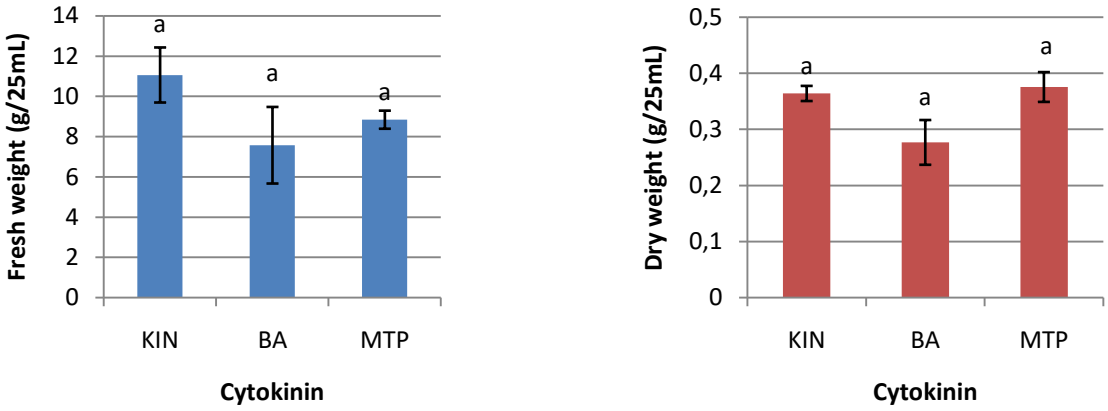


Figure 4. 36 Effect of equimolar concentration of different cytokinin on *S, tingitana* callus growth. Values represent the mean ± standard error (SE) n=4.

However, in order to optimize the *S. tingitana* callus biomass production, calli were cultured in MS containing KIN 2.32 µM and 10 mg/l ascorbic acid supplemented with equimolar concentration (4.52 µM) of two type of auxin (2,4-D and NAA). The maximum average of fresh and dry weights were observed when callus was cultured on medium containing 2,4-D as auxin with a production of 11.06 g of fresh or 0.363 g of dry weight compared to 3.38 g of fresh or 0.19 g of dry weight observed on medium supplemented with NAA (**Fig. 4.38**).

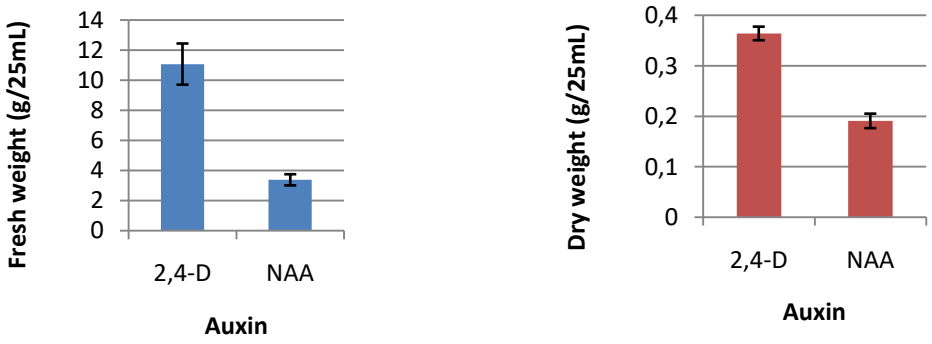


Figure 4. 37 Effect of equimolar concentration of 2,4-D and NAA on *S. tingitana* callus growth. Values represent the mean ± standard error (SE) n=4.

4.1.2.2.4. Growth kinetic of callus

The growth parameters (expressed as callus fresh and dry weight) of the callus established on MC medium (medium supplemented with 2,4-D 4.52 µM, KIN 2,32 µM and 10 mg /L of ascorbic acid) over a period of 5 weeks is shown in **Fig. 4.39**. *S. tingitana* callus maintained a high growth rate throughout the period of investigation with only a temporary slight growth reduction between the 3rd and the 4th week after callus formation. The callus fresh weight increased significantly during the last week reached a maximum growth with a weight growth index 323.5 %.

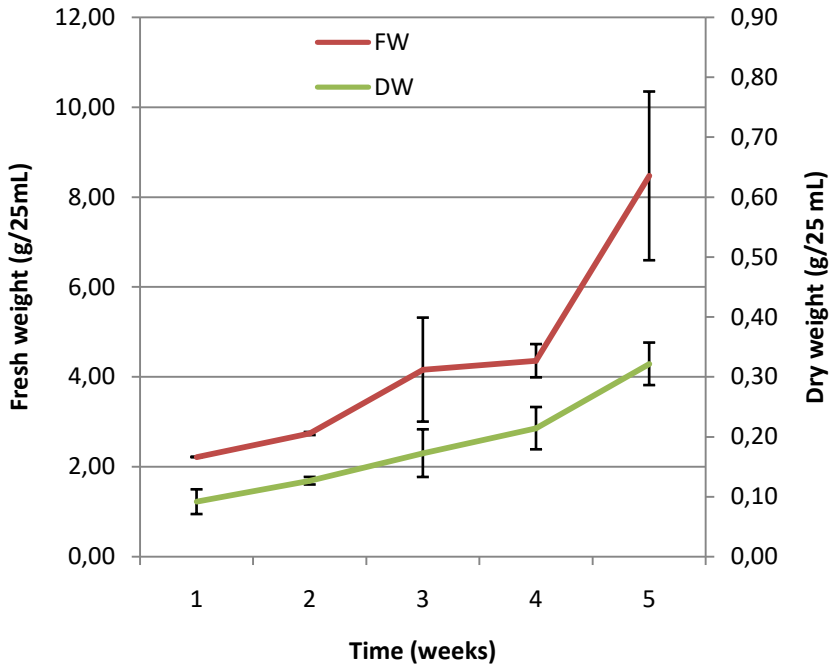


Figure 4. 38 *Salvia tingitana* callus growth curve over time on MS medium supplemented with 2,4-D 4.52 µM, KIN 2,32 µM and 10 mg/L of ascorbic acid (average ± SE) n=3.

4.1.2.2.5. Elicitation of *Salvia tingitana* callus

4.1.2.2.5.1. Elicitation with methyl jasmonate or light

Callus transferred on growth medium containing different concentration of MJ did not showed significative difference of fresh among the control and all the treatments. However, the MJ decreased the dry weight to the high concentration treatment (**Table 4.21**).

The effect of light elicitation for 2 weeks on *S. tingitana* callus did not show any difference of biomass production of callus growth in 16 hours photoperiod compared to calls in dark condition

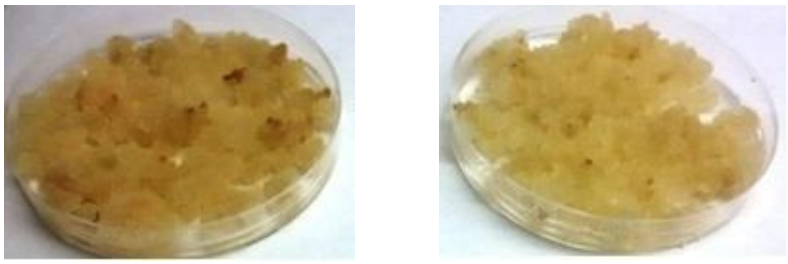
(**Table 4.22**). However, the aspect of callus in dark condition was more brown compared to callus in light (**Fig. 4.40**).

Table 4. 21 Effect of different concentrations of methyl jasmonate on fresh weight and dry weight of *S. tingitana* callus cultured on MC medium.

Treatment	FW ± SD	DW ± SD
Control	6.8 ± 1.3	0.23 ± 0.07 a
50 µM	5.7 ± 0.6	0.18 ± 0.06 ab
100 µM	5.4 ±0.3	0.13 ± 0.03 ab
200 µM	5.5 ± 0.4	0.10 ± 0.05 b

Table 4. 22 Effect of light elicitation for 2 weeks on *S, tingitana* callus growth. Values represent the mean ± standard deviation (SD) n=10

Treatments	FW	DW
dark	12.7 ± 1.6	0.34 ± 0.07
Light	12.3 ± 2.6	0.32 ± 0.05



0 h photoperiod 16 h photoperiod

Figure 4. 39 Aspect of *S. tingitana* callus after 2 weeks elicitation with light.

The TLC analysis of methanolic extracts did not showed any qualitative difference between the control and treated callus with MJ and Light (**Fig. 4.41**).

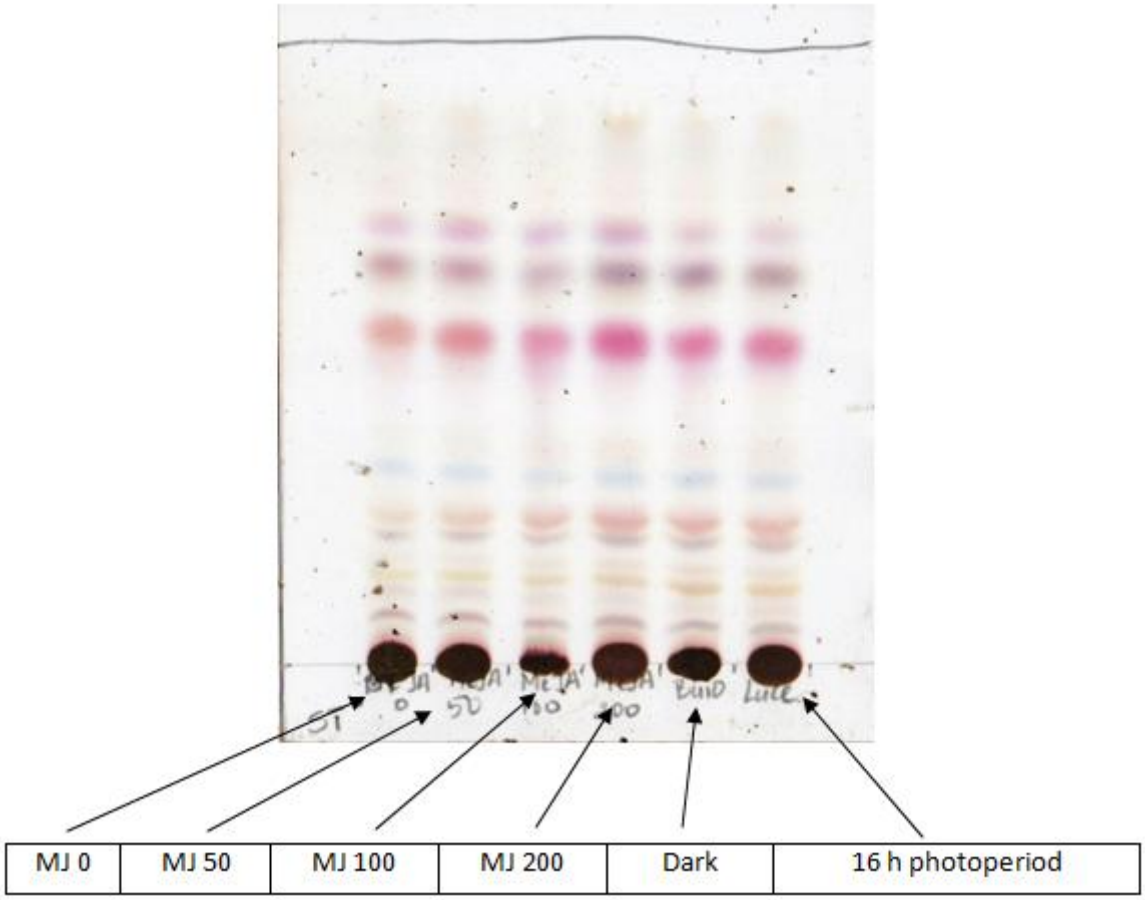


Figure 4. 40 TLC comparison *S. tingitana* callus elicitated with MJ (µM) or light.

4.1.2.2.6. Callus biomass production

The biomass production of *S. tingitana* callus onto 80 Petri containing MC medium in dark condition (**Fig. 4.42**) allowed to obtain a final biomass about 564 g or 26.62 g of dry weight, The callus fresh biomass is characterized by large contain of water, the dry biomass represented less than 5 % of fresh weight.

Figure 4. 41 Biomass production of *S. tingitana* callus into Petri dish at dark condition for 35 days.

4.1.2.3. Phytochemical analysis of *Salvia tingitana* Etl.

The methanolic extract of *S. tingitana* callus (7.5 g) was fractionated by Si gel MPLC (Medium Pressure Liquid Chromatography) to obtain 13 semi-purified fractions. The final TLC chromatograms from *S. tingitana* callus revealed several chemical compositions (**Fig. 4.43**). These fractions were dried at room temperature and allowed to obtain a dry mass represented in **Table 4.23**.

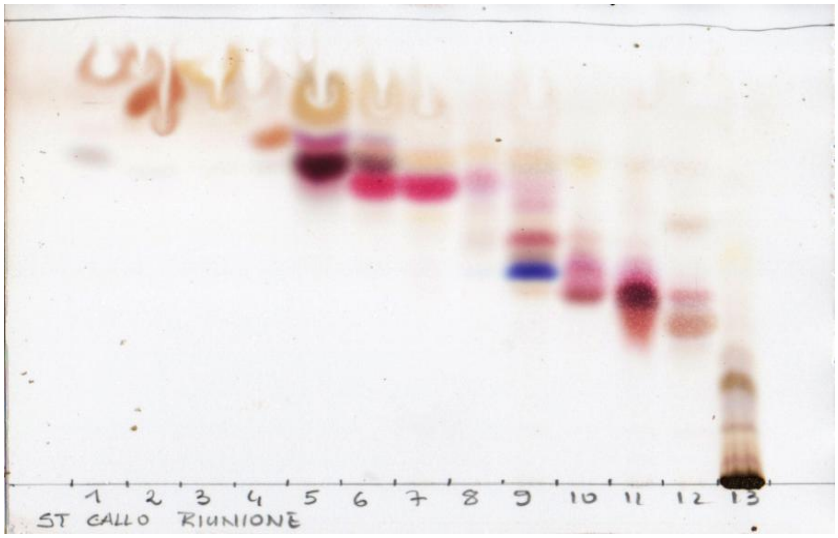


Figure 4. 42 TLC chromatogram of chemical composition of different fractions from *S. tingitana* callus culture extract.

Table 4. 23 Separation of methanolic extract of *S. tingitana* callus.

Fraction	Solvent	Initial fraction	Final fraction	Weight (mg)
1	n-hexane	1	8	11.6
2	CHCl3	9	15	28.5
3	CHCl3	16	22	34.5
4	CHCl3	23	26	18.3
5	CHCl3	27	29	101.9
6	CHCl3	30	32	60.4
7	CHCl3	33	41	43.8
8	CHCl3	42	42	21.8
9	CHCl3	43	45	57.8
10	CHCl3/MeOH 95:5	46	46	60.2
11	CHCl3/MeOH 95:5	47	49	78.9
12	CHCl3/MeOH 95:5	50	54	48.5
13	MeOH	55	57	5997.5

4.1.2.4. Antibacterial activity of *Salvia. tingitana* callus

The antibacterial activity of methanolic extract and fraction obtained after MPLC was reported in **table 4.24.** The methanolic extract of *S. tingitana* callus and huge amounts of fraction (1 to 10 and 13) are inactive (MIC value > 128 µg/mL) against all strains tested. The fraction 11 and 12 exhibited an antibacterial activity against *E. faecalis* and *E. faecium*. The best activity of against these stains was obtained with the fraction 11 (MIC value: 32 µg/mL).

E. coli and *S. aureus* were the most resistant strain against methanolic extract and all semi-purified fractions with all MIC values obtained greater than 128 µg/mL.

Table 4. 24 Minimal inhibitory concentrations (MICs) of *S. tingitana callus* methanolic extract and different fractions. MIC values are expressed in µg/mL.

strains	**MeOH extract**	**1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 13**	**11**	**12**
S.aureus	>128	>128	>128	>128
S. epidermidis	>128	>128	128	>128
E. faecalis	>128	>128	32	64
E. faecium	>128	>128	32	64
M. luteus	>128	>128	128	>128
E. coli	>128	>128	>128	>128

4.2. Discussion

4.2.1. Discussion of Salvia corrugata

In the literature of *Salvia* shoot elicitation, only *S. officinalis* was investigated using MJ by I. Grzegorczyk et al. (2009) and using sodium salicylate by Kračun-Kolarević et al. (2015). Unlike the slight increase of antioxidant (carnosol and carnosic acid) after elicitation with MJ reported by I. Grzegorczyk and Wysokińska (2009), our study showed a decrease of both fruticuline A and demethylfruticuline A in *S. corrugata* shoots after elicitation with MJ (**Table 4.3**). The values of demethylfruticuline A and fruticuline A present into the non treated shoots are almost similar to those obtained respectively on micropropagated plants and regenerated shoots by Bisio et al. (2016).

Integration of the T-DNA region with its genes in the plant genome is necessary for hairy root induction. In this study, the percentage of hairy root induction of *S. corrugata* Vahl. after cocultivation of leaves explants with the wild type *A. rhizogenes* ATCC 15834 was almost the double of that obtained by the hyper virulent strain *A. rhizogenes* LBA 9402 strain. According to our results, the percentage of 75 % obtained with *A. rhizogenes* ATCC15834 was relatively high compared to other *Salvia* species: 56.7 % on *S. officinalis* reported by Izabela Grzegorczyk et al. (2006) (Izabela Grzegorczyk et al., 2006), 20 % on *S. miltiorrhiza* reported by Zhi and Alfermann (1993), and 22 % on *S. wagneriana* obtained by Ruffoni et al. (2016). In opposite, the transformation of *S. corrugata* with the hypervirulent strain LBA 9402 obtained a relatively low rate 38.1% compared to 80% reported on *S. miltiorrhiza* (Zhi & Alfermann, 1993), and 100% obtained on *Ocimum basilicum* L. (D'Angiolillo et al., 2012). These authors estimated that it seems clear that there is a difference in the interaction between *Agrobacterium* strains and plant species, which *in vitro* is reflected in the different percentage of development of hairy root (D'Angiolillo et al., 2012).

The fundamental basis of and efficient hairy root culture process is the development of an appropriate hairy root clone line that maximizes both growth and rate and product yield (Davies et al., 2014). The difference of growth and branching behaviour observed among clones might to be due to variations in the present and expression of *rol A, B* and *C* genes for individual lines, which could alter the biosynthesis of endogenous growth regulators or the sensitivity of plant cells to

growth regulators. The metabolic and growth kinetics differences in HR lines depend to the site of integration of T-DNA into the plant genome (Hu et al., 2006).

In the scale up process, in our study, after 42 days, hairy root showed at the central dark brown color, which lacked viability and at the external part clear and vital roots that begun to turn brown. This behavior may be related to the decrease of nutritive compounds in the medium that bring to ageing and/or cell death; this is also observed by Kuzma et al. (2008) of *S. sclarea* hairy roots after 30 days, culture.

The hairy root growth can be easily and indirectly monitored by measurement of the conductivity. The decrease in conductivity can be attributed to the intake of nutrients by growing hairy roots leading to a decrease in the number of ions. An inverse relationship between biomass and conductivity was showed by Taya et al. (1989) This behavior was observed not only by our research team on *S. corrugata* hairy root, but also by Urbanska et al. (2014) on *Platycodon grandiflorum* hairy roots growth.

Media composition could have a significant impact on hairy root growth in culture systems (Sivakumar et al., 2005). In present study it has been shown that, media MS0 and ½ WPM achieved both the best *S. corrugata* hairy roots dry biomass production.

Sucrose represents the principal carbon source in plant culture due to its effective absorption through the cell membrane (Bozena et al., 1991). The determining role of sucrose concentration on biomass production yield of hairy roots culture has been recognized comprehensively (A. Giri et al., 2000). According to H. Chen et al. (1997), initial sucrose concentration had a significant influence on the growth of transformed cell cultures of *S. miltiorrhiza*; showing the fastest growth in medium containing 30 g/L sucrose. This concentration was reported to be the best for *Arnica montana* hairy root growth by (Petrova et al. (2015)). The similar value of sucrose 30 g/l obtained in this study was found to promote of the *S. corrugata* hairy root biomass production (**Fig. 4.19**). The current result is consistent with the pattern for the *in vitro* activity emerging from other studies. In some cases, the increase of carbon concentration leads reduction of biomass. This reduction can be caused by an excessive osmotic contribution or by toxicity of the carbohydrate (Ślesak et al., 2004). Some authors reported this negative effect of high sucrose concentrations: Thiruvengadam et al. (2014) on hairy roots of bitter melon (*Momordica charantia* L.) and H. Chen et al. (1997) on transformed cell suspension cultures of *S. miltiorrhiza.*

Many studies report that to find the specific concentration of the elicitor plays a very important role in elicitation process. A low concentration can be insufficient to stimulate secondary metabolite and a high concentration can decrease the growth of biomass or cause the death of plant. However, the initial rapport between hairy root fresh mass and culture medium volume could positively or negatively influence biomass accumulation. Our experimental results show that the biomass accumulation in *S. corrugata* hairy roots can be stimulated by both biotic and abiotic elicitors with high levels. Similar positive effect of Yeast extract (YE) on biomass production was found by eliciting hairy roots of *Salvia castanea* Diels f. *tomentosa* Stib. (Hui Chen et al., 2001; D. Yang et al., 2018) and *S. miltiorrhiza* (Bo Li et al., 2016). However, our result showed a slight inhibition of biomass production with low concentration of YE. This result is consistent with those previously reported by other authors on the *S. miltiorrhiza* hairy roots (Ming Shi et al., 2007; Jian-Yong Wu & Shi, 2008).

In this study, the cultivation of *S. corrugata* hairy root in TIS RITA® bioreactor allowed to obtained considerable fresh and dry biomass, however, the non-homogenous repartition and the formation of clump complicated the biomass production for long time and the process of optimization; the similar observation was reported by (M. I. Georgiev et al., 2007). It was noticed in this work that all the biomass of different clones produced by the design of the RITA® bioreactor allowed to minimize the stress of hairy root and avoid the callus formation. Generally, bioreactors with the high shear stress caused by a mechanical agitation of liquid-phase (impeller rotation) such as STBs (Stirred-Tank Bioreactors) are not suitable for hairy roots culture because of the wound response and callus formation (Mishra et al., 2008).

Some synthetic pathways are specifically located in root or shoot, there are some differences in the metabolic profiles of the two organs even if they come from the same genotype (Subroto et al., 1996). We found that the hairy root culture of *S. corrugata* did not produce antibacterial diterpenoids present in the area part on the plant such as fruticuline A and demethylfruticuline A. This tissue specificity behavior of secondary metabolite production was also demonstrated on the production of carnosol and carnosic acid into the aerial part and absent into the normal and transformed root of *S. officinalis* (Izabela Grzegorczyk et al., 2006).

The integration of A. rhizogenes DNA into the plant genome may produce new compounds that normally are not present in intact plants (Sheludko et al., 2002). In our study, the purification of one fraction of *S. corrugata* hairy root extract allowed to identified a new bioactive diterpene compound (19[4→3]abeo-O-demethyl-14-hydroxy-cryptojaponol). In accordance with our result, it has been shown in the past the production of a new bioactive taxodione derivative from hairy roots of *Salvia austriaca* by (Łukasz Kuźma et al. (2012); Wysokińska et al. (2012)), and the production of two new abietane dimers from cultures of transformed roots of *Salvia broussonetii* by (Fraga et al., 2005).

The diterpenes quinone horminone and 7-acetyl-horminone isolated from our MeOH extract were previously isolated from *S. blepharochlaena* and were displayed an antibacterial actictity against *Staphylococcus* and *Enterococcus* strains (Ulubelen et al., 2001). These compounds were successively purified from roots of *S. officinalis* and *S. ringens* and were found to be active as antitumor agents (Janicsák et al., 2011; Slameňová et al., 2004).

4.2.2. Discussion of *Salvia tingitana*

In the present study, among the different sterilization method of *S. tingitana* explants, the pre-treatment with tap water for 15' combined with NaClO sterilization with the concentration of 1 % or 2 % for 15' was the best to maintain the viable and uncontaminated shoots.
The pre-treatment with ethanol 70 % (v/v) for 1 min totally inhibit the shoot viability; this result is in opposite to that obtained in a lot of other species for example by Ruffoni et al. (2009), in *C. maritimum* or by Pistelli et al. (2013) in *M. communis.*

In our study *S. tingitana* seeds germinated at 40%. This percentage is lower than the 70 % reported in *in vivo* germination of *S. tingitana* by Cervelli (2004).

The explants of *S. tingitana* leaves placed on medium without PGRs or only with KIN didn't show callus induction in dark or light condition; moreover, high concentration of 2,4-D inhibited the induction only in light condition. On the contrary, culture in dark permitted to develop callus with all the other substrates used. All those suggest that 2,4-D and dark play a crucial role in callus induction. This is in accordance with Zare et al. (2015), that reported that callus formation in *Linumglaucum* was best induced with the medium containing 2,4-D 0.5 mg/L and was higher in dark than in light condition. Many investigators attempted to establish callus cultures from various *Salvia* species using a combination of 2,4-D and BA at various concentrations.

The selection of suitable combination of exogenous auxin and cytokinin is a perquisite to ensure the suitable amount that maintains cell division and expansion. The external supply of plant growth regulators must work in cooperation with endogenous plant hormones to sustain cell growth and development. In this study, the behavior of the *S. tingitana* fresh callus growth curve over time on MS medium supplemented with 2,4-D 4.52 µM, KIN 2,32 µM and 10 mg/L of ascorbic acid is mostly similar to that obtained on *S. fruticosa* callus cultures on MS medium supplemented with 4.5 mM 2,4-D and 4.5 mM KIN (Kintzios et al., 1999).

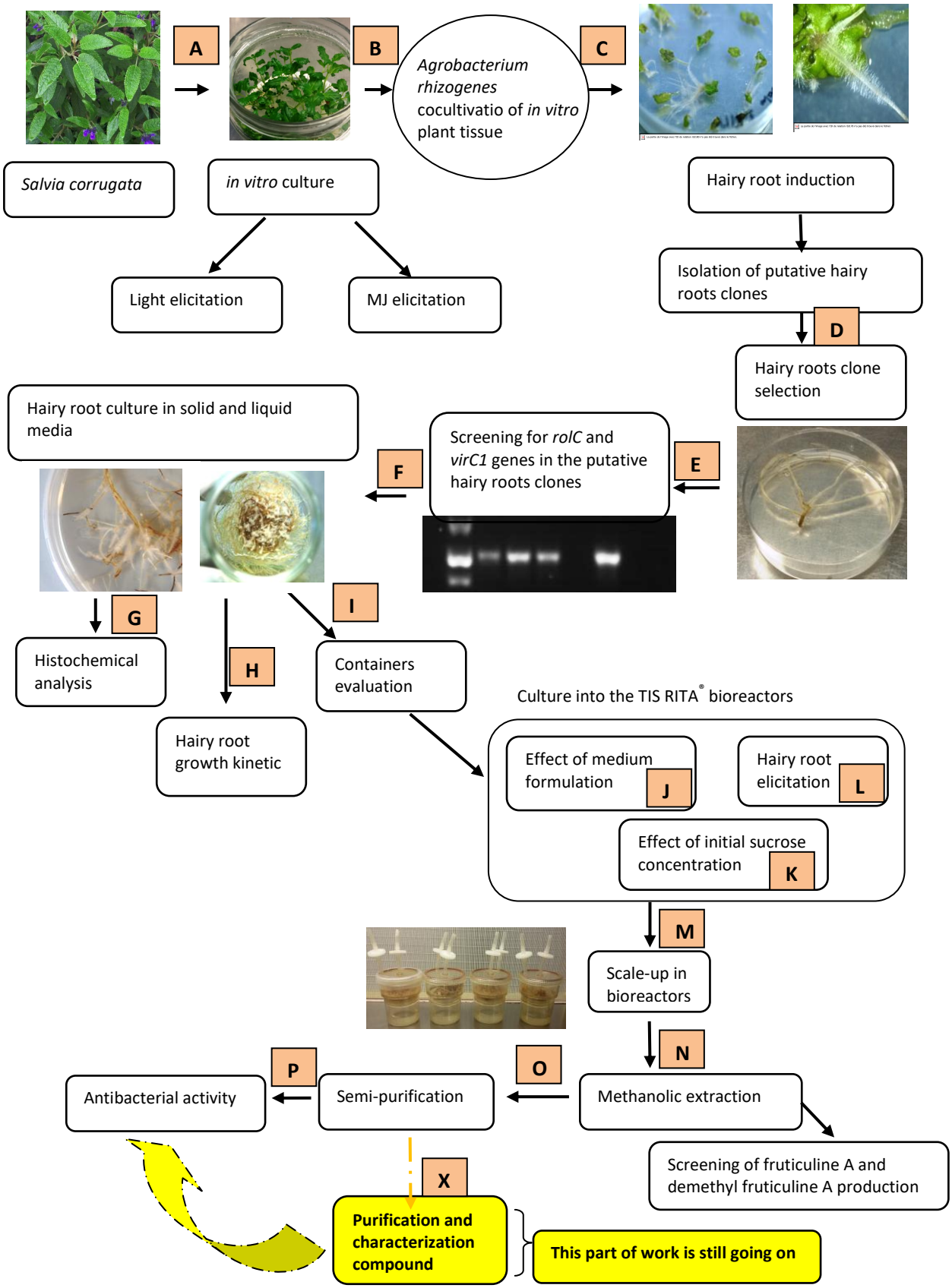
Protocols for calli cultures have been already established for many *Salvia* species with the aim to evaluate the content of secondary metabolites. It is accepeted that the dedifferentiation of plant tissus during the establishment of callus is often connected with the reduction of secondary metabolite (Izabela Grzegorczyk et al., 2005; Marco Savona et al., 2017).
The results on antimicrobial activity showed that the tested micro-organisms revealed variable responses to different S. tingitana callus fractions. The crude methanolic extract of S. tingitana

callus and huge amounts of fraction (1 to 10 and 13) are inactive (MIC value > 128 µg/mL) against all strains tested. Similar results were reported by Bisio et al. (2016) on methanolic extract of *S. corrugata* callus and by Vineet et al. (2010) on ethanolic extract of Plumbagozeylanica callus showing the mic values of 5000 µg/mL. Although the methanolic extract and some fractions S. tingitana callus were inactive to inhibit the growth of most studied bacterial, two fraction active. The antibacterial activity of these two fractions can be accredited to the presence of bioactive compounds that are not still known. Thereafter, isolation and identification of bioactive compounds in the active fractions is indispensable.

CHAPTER 5 SUMMARY AND CONCLUSION

S. corruagata

Important findings of different experiments of the *S. corrugata* study are visually summarized below:



STEPS

A- The shoot explants of *S. corrugata* grown in the greenhouse were sterilized with NaClO and inoculated onto solid MSO medium to obtain sterile *in vitro* shoots.

B- The sterile leaf explants were cocultivated with two strains of *A. rhizogenes* (wild type ATCC 15834 and hypervirulent LBA 9402) in order to obtain hairy roots.

C- The explants infected with *A. rhizogenes* ATCC 15834 showed the high induction rate 75% while the explants exposed to *A. rhizogenes* LBA 9402 showed 38% of induction after 30 days of coculture.

D- The selection of clones for biomass production was based on evaluation of length increase, and branching index. Two putative hairy root clones (SCO-HR-FA8 and SCO-HR-FA13) were selected from roots obtained from ATCC 15834 and one clone SCO-HR-FL7 from roots obtained from LBA 9402.

E- The investigation of integration of T-DNA on selected clone was carried out using *rolC* and *VirC1* gene. The results reported that the three selected clones were really transformed by the respective *A. Rhizogenes* strains.

F- The selected root clones were investigated to determine the ability to growth in liquid medium. A sufficient quantity was produced to begin the biomass scale up process.

G- The histochemical analyses were carried out on with Nadi staining and the viability test. The Nadi allowed us to highlight the presence of terpenes droplet into the hair of root. The test of viability was positive both with the FDA and calcein AM stainings.

H- The growth kinetic showed a classical sigmoid curve with the three principal growth phases: latent, exponential and plateau. This experience showed the useful interval of time in which the hairy root could be elicitated.

I- The container evaluation between the glass vessel and bioreactor for biomass production did not show any significant difference.

J- The evaluation of medium formulation allowed to identify the MS and ½ WPM as the best media formulation for the biomass production.

K- The best growth was observed when hairy roots were cultivated in the MS0 medium containing 30 g/l sucrose

L- The application of elicitation allowed to show the optimal dose depending on biomass production.

M- The scale up of biomass production using the RITA bioreactor resulted in significant material amount, fresh and dry, required for phytochemical analysis.

N- The dry material was extracted through maceration with methanol

O- The methanolic extract was semi purified by Flash chromatography to obtain 16 semi purified fractions

P- The methanolic extract and semi purified fractions were tested to determine the antibacterial activity. Four semi purified fractions showed MIC values ranging from 8 to 128 µg/mL against *Staphylococci* and from 4 to 64 µg/mL against *Enterococci.*

X- This part of work is still going on

Concluding this part:

It may be concluded that, the protocol developed to obtain *in vitro S. corrugata* hairy root culture, and the biomass production in bioreactor is a successfully approach to produce plant material and antibacterial compounds. However not all the compound present into the whole plant can be producing using this technology. Certain semi-purified fractions exhibit interesting antibacterial activity, so that, future prospects of purification may lead to the discovery of pure compounds with strong antibacterial activity. It is therefore interesting to complete our investigation with the isolation and characterization of compound present in these fractions, which showed good antibacterial activity. It will also be interesting to determine the capacity of the *S. corrugata* hairy roots to produce metabolites different from those spontaneously produced by the whole plant.

S. tingitana callus

Callus culture of *S. tingitana* (Lamiaceae) was successfully established for the first time from in vivo sterile explants. The effects of light illumination and plant growth regulators on the initiation of callus were investigated and were shown to affect significantly both the formation and the quality of callus. The condition suitable for initiation of callus from leaf explant was MS

medium supplemented with 0.5 mg/L of KIN and 0.5 mg/L of 2,4-D, and 1 mg/L of KIN and 1 mg/L of 2,4-D added of 10 mg/L ascorbic acid, while the best callus growth was observed in MS medium supplemented with 0.5 mg/L of KIN and 1 mg/L of 2,4-D, after 5 weeks. The material is characterized by high accumulation of water more than 95 %. TLC analysis of methanolic extract of dry weight showed that callus tissues retained the ability to accumulate secondary metabolites and the application of MJ and Light elicitation did not allow the production of different other metabolites. Two semi purified fraction displayed an antibacterial activity with MICs values ranging from 32 to 64 µg/mL against *E. Faecalis and E. faecium*. Also here, it is therefore interesting to complete our investigation with the isolation and characterization of compound present in these fractions, which showed good antibacterial activity. It will also be interesting to determine the capacity of *S. tingitana* callus to produce metabolites different from those spontaneously produced by the whole plant.

Finally, it may be conclude that *in vitro* cultures of callus and hairy root of *S. corrugata* and *S. tingitana* can be used as another method to obtain the plant materials in controlled environment, as well as the bioactive compounds for the applications in pharmaceuticals, nutritionals and cosmetics.

Annex

Annex 1. Composition of different base medium for plant growth in 1 Liter

Medium composition (mg/L)	MSO	B5	WPM	67-V
Macronutrients				
NH_4NO_3	1650	-	400	100
$(NH_4)_2SO_4$	-	134	-	
$Ca(NO3)_2.4H_2O$	-	-	556	
KNO_3	1900	2500	-	800
K_2SO_4	-	-	990	
$CaCl_2.2H_2O$	440	150	96	200
$MgSO_4.7H_2O$	370	500	370	250
KH_2PO_4	170	-	170	
$NaH_2PO_4.H_2O$	-	150	-	
NaH2PO4.2H2O	170	-	-	
NaH_2P0_4	-	-	-	150
Na_2HP0_4	-	-	-	20
Micronutrients				
KI	0.83	0.75	-	0.05
H_3BO_3	6.2	3.00	6.2	5.0
$MnSO_4.4H_2O$	22.3	-	-	
$MnSO_4.H_2O$	-	10.0	22.3	4.0
$ZnSO_4.7H_2O$	8.6	2.0	8.6	1.5
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	-	0.25	0.25
$CuSO_4$	-	0.025	-	
$CoCl_2.6H_2O$	0.025	0.025	-	0.25
$Na_2EDTA\ 2H_2O$	37.3	-	37.3	18.6
$FeSO_4.7H_2O$	27.8	-	27.8	13.9
Iron	-	28	-	
Organic compounds				
Inositol	100	100	100	100
Ca-panthotenate	-	-	-	1.0
Gycine	2.0	-	2.0	
thiamine HCl	0.1	10.0	1.0	0.5
Pyridoxine HCl	0.5	1.0	0.5	0.5
Nicotinic acid	0.5	1.0	0.5	1.25
Casein hydrolysate	-	-	-	2
pH	5.8	5.5	4.5	4.5

Annex 2. Dneasy Plant mini Kit (Qiagen®): Quick-Start Protocol

Notes before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- If necessary, redissolve any precipitates in Buffer AP1 and Buffer AW1 concentrates.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates.
- Preheat a water bath or heating block to 65°C.

1. Disrupt samples (≤100 mg wet weight or ≤20 mg lyophilized tissue) using the TissueRuptor®, the TissueLyser II or a mortar and pestle.

2. Add 400 µl Buffer AP1 and 4 µl RNase A. Vortex and incubate for 10 min at 65°C. Invert the tube 2–3 times during incubation.

3. Add 130 µl Buffer P3. Mix and incubate for 5 min on ice.

4. Recommended: Centrifuge the lysate for 5 min at 20,000 x *g* (14,000 rpm).

5. Pipet the lysate into a QIAshredder spin column placed in a 2 ml collection tube. Centrifuge for 2 min at 20,000 x *g*.

6. Transfer the flow-through into a new tube without disturbing the pellet if present. Add 1.5 volumes of Buffer AW1, and mix by pipetting.

7. Transfer 650 µl of the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge for 1 min at ≥6000 x *g* (≥8000 rpm). Discard the flow-through. Repeat this step with the remaining sample.

8. Place the spin column into a new 2 ml collection tube. Add 500 µl Buffer AW2, and centrifuge for 1 min at ≥6000 x *g*. Discard the flow-through.

9. Add another 500 µl Buffer AW2. Centrifuge for 2 min at 20,000 x *g*.

10. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

11. Add 100 µl Buffer AE for elution. Incubate for 5 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x *g*.

12. Repeat step 11.

Note: Do not mix Buffer AP1 and RNase A before use.

Note: Remove the spin column from the collection tube carefully so that the column does not come into contact with the flow-through.

Annex 3. Klimiuk method: plasmid extraction from Agrobacterium

From sterile Eppendorf tubes, containing suspension of *A. rhizogenes*

- Centrifuged and eliminated the supernatant
- Watched with LB media (added LB, centrifuged and eliminated the supernatant)
- Re-suspended a colony of Agrobacterium inside 20 µL of LB
- Added 40 µI of 0.25 M NaOH to the sample and incubated in a boiling water bath for 30 sec
- Subsequently neutralized by addition of 40 µL 0.25 M HCI and 20 µL Tris-HCI Nonidet (Sigma) 0.25% (V/V), 0.5 M PH 8.0, before boiling for a further 2 min.
- Stored the sample at + 4 °C
- Before using the sample (if previously stored in the fridge) boiling for 2 min
- 1 µL wasused for PCR

Note: The critical step in the procedure is the initial period of boiling in 0.25 M NaOH;

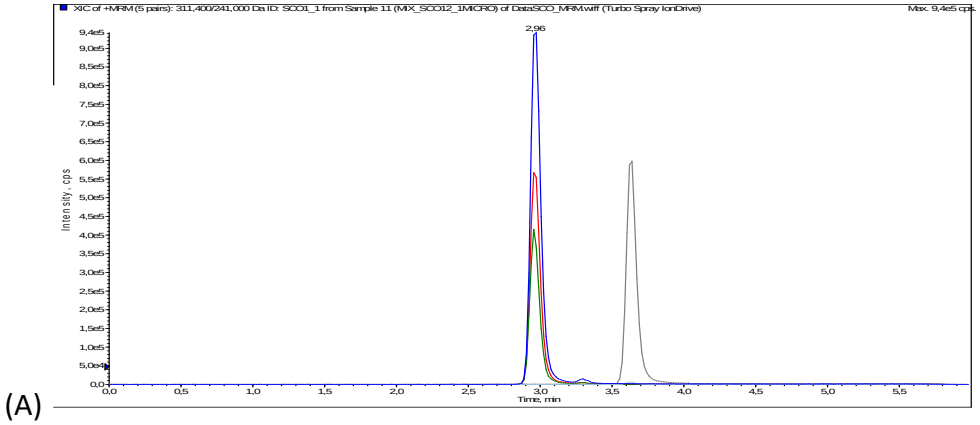
Luria Bertani (**LB**) medium formulation per one liter: 10 g Peptone 140, 5 g Yeast Extract, 5 g sodium chloride, pH 7.0.

Annex 4. Electrophoresis gel preparation

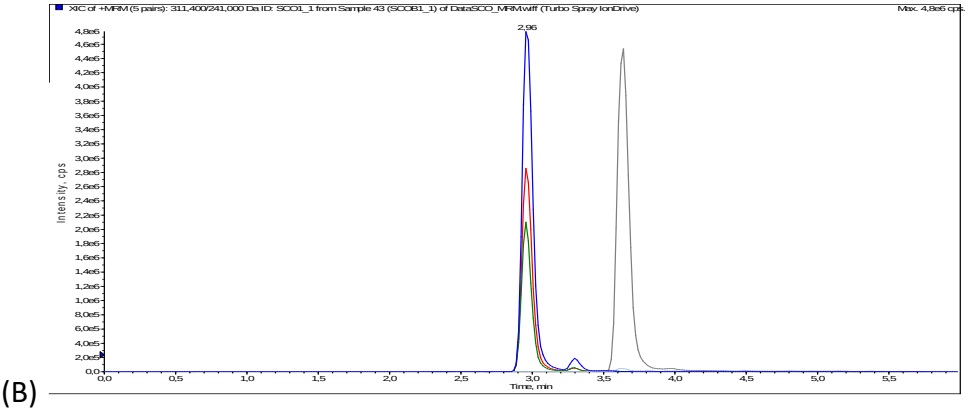
- Mixture of 0.6 g of agarose powder with 40 mL of TAE (Tris-acetate EDTA buffer: Tris-acetate 0.045 M and EDTA 0.001 M) 1%
- Heat the suspension in the microwave avoiding boiling until the transparent solution is formed.
- leave the solution to stir until cool to a temperature of about 40 degrees
- Add 1.5 µL of ethidium bromide
- Pouring the gel in tray used for electrophoresis and leave solidification.

Annex 5

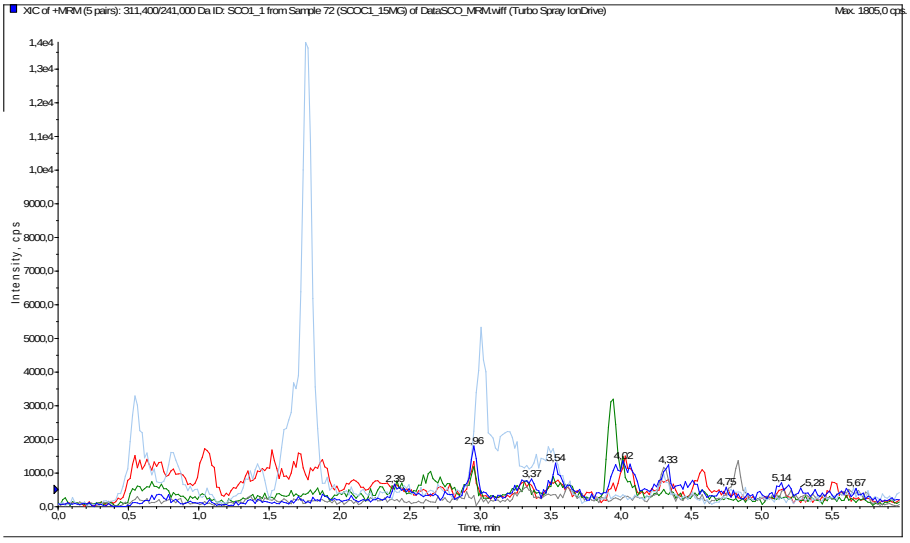
Representative chromatograms of the LC-MS/MS (MRM) profile of fruticuline A and demthylfruticuline A. (A) standards (RT 2.96 and 3.63), (B) methanolic extract of *S. corrugata* shoot (control), (C) methanolic extract of hairy roots of *S. corrugata.*



(A)



(B)

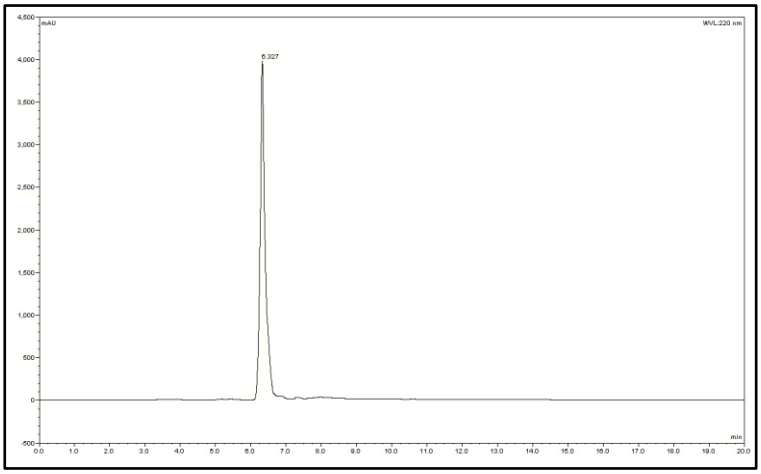


(C)

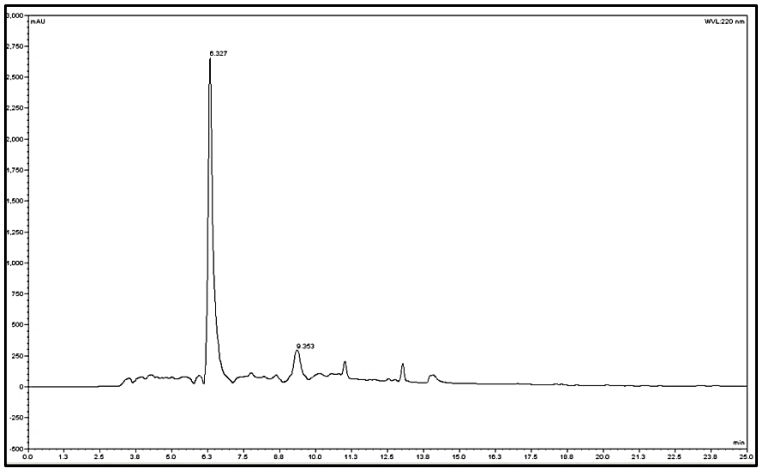
Annex 6

Representative chromatograms of the HPLC-PDA profiles of (a) demethylfruticuline A (RT 6.327), (b) the methanolic extract of shoots of *S. corrugata* at 220 nm (Bisio et al., 2016).

(a)



(b)



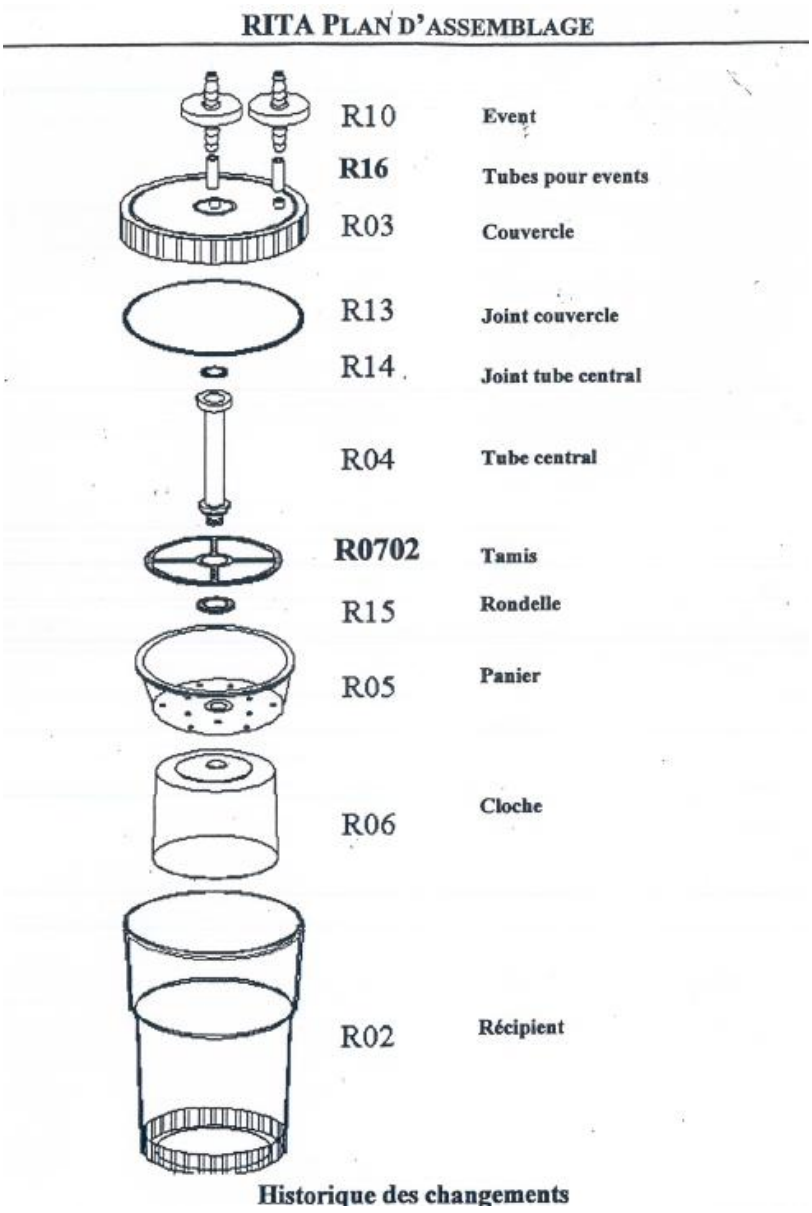


Diagram of the RITA® bioreactor assembly

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